Mitofusin 2-Containing Mitochondrial-Reticular Microdomains Direct Rapid Cardiomyocyte Bioenergetic Responses Via Interorganelle Ca\textsuperscript{2+} Crosstalk

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**Rationale:** Mitochondrial Ca\textsuperscript{2+} uptake is essential for the bioenergetic feedback response through stimulation of Krebs cycle dehydrogenases. Close association of mitochondria to the sarcoplasmic reticulum (SR) may explain efficient mitochondrial Ca\textsuperscript{2+} uptake despite low Ca\textsuperscript{2+} affinity of the mitochondrial Ca\textsuperscript{2+} uniporter. However, the existence of such mitochondrial Ca\textsuperscript{2+} microdomains and their functional role are presently unresolved. Mitofusin (Mfn) 1 and 2 mediate mitochondrial outer membrane fusion, whereas Mfn2 but not Mfn1 tethers endoplasmic reticulum to mitochondria in noncardiac cells.

**Objective:** To elucidate roles for Mfn1 and 2 in SR-mitochondrial tethering, Ca\textsuperscript{2+} signaling, and bioenergetic regulation in cardiac myocytes.

**Methods and Results:** Fruit fly heart tubes deficient of the Drosophila Mfn ortholog MARF had increased contraction-associated and caffeine-sensitive Ca\textsuperscript{2+} release, suggesting a role for Mfn in SR Ca\textsuperscript{2+} handling. Whereas cardiac-specific Mfn1 ablation had no effects on murine heart function or Ca\textsuperscript{2+} cycling, Mfn2 deficiency decreased cardiomyocyte SR-mitochondrial contact length by 30\% and reduced the content of SR-associated proteins in mitochondria-associated membranes. This was associated with decreased mitochondrial Ca\textsuperscript{2+} uptake (despite unchanged mitochondrial membrane potential) but increased steady-state and caffeine-induced SR Ca\textsuperscript{2+} release. Accordingly, Ca\textsuperscript{2+}-induced stimulation of Krebs cycle dehydrogenases during \beta-adrenergic stimulation was hampered in Mfn2-KO but not Mfn1-KO myocytes, evidenced by oxidation of the redox states of NAD(P)H/NAD(P)\textsuperscript{+} and FADH\textsubscript{2}/FAD.

**Conclusions:** Physical tethering of SR and mitochondria via Mfn2 is essential for normal interorganelle Ca\textsuperscript{2+} signaling in the myocardium, consistent with a requirement for SR-mitochondrial Ca\textsuperscript{2+} signaling through microdomains in the cardiomyocyte bioenergetic feedback response to physiological stress. (Circ Res. 2012;111:863-875.)

**Key Words:** calcium signaling ■ cardiac metabolism ■ excitation-contraction coupling ■ mitochondria ■ redox
dative phosphorylation, where the Krebs cycle produces NADH and FADH\textsubscript{2} that fuel the electron transport chain (ETC). This electron flux generates the mitochondrial membrane potential (\(\Delta \Psi m\)), which is the driving force for ATP production at the F\textsubscript{1}F\textsubscript{0}-ATPase, but also for Ca\textsuperscript{2+} uptake into mitochondria via the mitochondrial Ca\textsuperscript{2+} uniporter (MCU). Ca\textsuperscript{2+} is a key regulator of oxidative phosphorylation by stimulating rate-limiting enzymes of the Krebs cycle, increasing the availability of NADH and FADH\textsubscript{2} for the electron transport chain.\textsuperscript{6,7} Yet, the role of mitochondrial fusion proteins in cardiac homeostasis is currently unresolved because the highly ordered subcellular architecture of cardiac myocytes physically enforces intermitochondrial connectivity, which may obviate the need for molecular tethering.\textsuperscript{8} Recent studies suggest that mitochondrial fusion and fission occur in the heart,\textsuperscript{9} and essential roles for mitochondrial fusion with functional overlap of Mfn1 and Mfn2 have been described in mitochondrial assembly regulatory factor (MARF) RNAi Drosophila heart tubes and in mfn1/mfn2 double cardiac-specific knockout murine hearts.\textsuperscript{10,11}

The conventional view that mammalian Mfn1 and Mfn2 are largely functionally redundant has been challenged by de Brito and Scorrano’s discovery that Mfn2 but not Mfn1 bridges mitochondria and endoplasmic reticulum (ER).\textsuperscript{12} Tethering of ER to mitochondria is thought to maintain close associations between the organelles and facilitate local Ca\textsuperscript{2+} delivery to the mitochondrial matrix,\textsuperscript{13} promoting mitochondrial Ca\textsuperscript{2+} signaling.\textsuperscript{13} Consistent with Mfn2 functioning as the ER-mitochondrial tether, ablation or suppression of Mfn2 (but not Mfn1) in murine embryonic fibroblasts and HeLa cells increased the spatial separation between ER and mitochondria, augmented ER Ca\textsuperscript{2+} content, and decreased mitochondrial Ca\textsuperscript{2+} uptake after inositol-trisphosphate (IP\textsubscript{3}) stimulation. These results established the molecular components of conceptual ER-mitochondrial Ca\textsuperscript{2+} microdomains originally proposed by Rizzuto and Pozzan.\textsuperscript{14}

In contrast to noncardiac cells, in which the concept of mitochondrial Ca\textsuperscript{2+} microdomains is now well established,\textsuperscript{14} their existence and functional implications in cardiac myocytes are still unclear.\textsuperscript{7,15–17} In this context, a recent report from Papanicolaou et al calls into question de Brito and Scorrano’s findings as they apply to cardiac myocytes.\textsuperscript{18} Cardiac-specific ablation of mfn2 induced mitochondrial enlargement and cardiac hypertrophy in otherwise normal hearts without apparently altering the interaction between mitochondria and sarcoplasmic reticulum (SR) or affecting cardiomyocyte Ca\textsuperscript{2+} cycling.\textsuperscript{18} Although Mfn2 deficiency in this study protected cardiomyocytes against mitochondrial depolarization and programmed cell death induced by reactive oxygen species (ROS) as predicted by de Brito and Scorrano,\textsuperscript{12} this was attributed to an intrinsic increase in mitochondrial Ca\textsuperscript{2+} retention capacity and decreased sensitivity of the mitochondrial permeability transition pore, and not to altered mitochondrial-SR interactions.\textsuperscript{18} The differences between de Brito and Scorrano’s findings in fibroblasts\textsuperscript{13} and those of Papanicolaou et al in mouse hearts\textsuperscript{19} suggest several possibilities: First, SR-mitochondrial bridges in cardiac myocytes are not created by Mfn2 but consist of other protein tethers such as PACS-2 and/or IP\textsubscript{3} receptor-VDAC complexes.\textsuperscript{19,20} Second, Mfn2 can bridge cardiac myocyte SR and mitochondria, but this physical tethering is functionally unimportant either because of enforced proximity of these organelles in cardiac cells or because mitochondrial sensing of SR Ca\textsuperscript{2+} released through ryanodine receptors (RyR) differs from that released via IP\textsubscript{3} receptors.\textsuperscript{21} Finally, Mfn2 SR-mitochondrial bridges may exist in cardiac myocytes to serve the purely pathological function described by Papanicolaou et al,\textsuperscript{14} increasing mitochondrial sensitivity to Ca\textsuperscript{2+}-mediated depolarization and cell death without altering normal SR or mitochondrial Ca\textsuperscript{2+} handling.

We address these possibilities using a previously described cardiac-specific Drosophila MARF (the Drosophila mitofusin ortholog) RNAi model\textsuperscript{10} and novel murine Mfn1 and Mfn2 knockout models in which mitofusin ablation is induced after birth without confounding toxic effects of the highly expressed Cre transgene used by Papanicolaou et al.\textsuperscript{22} Our results are consistent with the idea that Mfn2 is an essential component of the physical connections linking mouse cardiomyocyte SR and mitochondria. Disruption of these interorganelle tethering by Mfn2 ablation significantly increased caffeine-induced SR Ca\textsuperscript{2+} release and steady-state cytosolic Ca\textsuperscript{2+} transients. By simultaneously assaying mitochondrial ([Ca\textsuperscript{2+}]\textsubscript{m}) and cytosolic Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]\textsubscript{c}) in “beating” cardiac myocytes and monitoring substrates for oxidative phosphorylation, we further show that interrupting SR-mitochondrial Ca\textsuperscript{2+} crosstalk depresses mitochondrial Ca\textsuperscript{2+} and bioenergetic responses to increased work. Thus, we conclude that Mfn2 is an essential component of cardiomyocyte SR-mitochondrial contact points and that Ca\textsuperscript{2+} microdomains maintained by Mfn2-mediated SR-mitochondrial tethering are required in the heart to acutely adjust mitochondrial bioenergetic activity to instantaneous metabolic demand.

**Methods**

**Mouse Generation and Phenotypic Analyses**

Mfn\textsuperscript{1loxp/loxp} and Mfn\textsuperscript{2loxp/loxp} mice\textsuperscript{23} were obtained from University of California-Davis and crossed onto the Myh6 nuclear-directed “turbo” Cre line\textsuperscript{24} for cardiomyocyte-specific gene deletion after birth. Noninvasive assessment of left ventricular (LV) chamber size and ejection performance used M-mode echocardiography performed
on unsedated mice. Physiological measurements were performed on 6- to 8-week-old mice. Invasive hemodynamic evaluation of LV contractile function and the response to atrial pacing or infused β₁-adrenergic agonist dobutamine was performed using standard techniques as described. Analysis of isolated murine ventricular myocyte cell shortening and Ca²⁺ signaling was performed as described.²²⁻²⁶ All experimental procedures were approved by the Animal Studies Committee at Washington University School of Medicine.

**Drosophila Models and Phenotypic Analyses**

The dMFN/MARF RNAi fly line used in this study was provided by Ming Guo.¹⁰,²⁷ Rolf Bodmer (Sanford-Burnham Medical Research Institute, La Jolla, CA) provided the tinc4-A-Gal4 stock.²⁸ The GCaMP3.0 expressing *Drosophila* line was obtained from the Bloomington Stock Center (stock No. 32234). In situ analysis of working heart tube dimension and contraction by optical coherence tomography was as described.¹⁰ Ca²⁺ signal measurement from in situ heart tubes expressing GCaMP3.0 was performed on semi-intact 3-day-old adult flies. Flies were dissected and maintained in artificial hemolymph.²⁹ Cuts were made anterior to the abdomen, removing the head thorax and legs in one cut. The posterior abdominal segments were likewise removed. Lateral cuts along the abdominal cuticle were made on each side of the heart-tube. These cuts allowed for the removal of the ventral portion of the abdomen and revealed the beating heart tube. To control for variability in heart rate between animals, specimens were kept on slides suspended over an ice bath chilled to 10°C. Phasic Ca²⁺ transients were captured over a 10-second period on a Nikon AZ1000 UV fluorescent microscope at ×100 magnification. Caffeine (10 mmol/L) was added to nifedipine-arrested (300 µmol/L) heart tubes to stimulate full SR Ca²⁺ export. Change in fluorescence was measured over a 150×100 pixel area centered over the conical region of the heart tube. Images were acquired at ~150 frames per second using a Photometrics Evolve EMCCD camera. Image analysis was performed with Nikon NIS elements 3.0 Advanced software.

**Ultrastructure Studies by Transmission Electron Microscopy**

A detailed description of these studies is given in the Online Supplement.

**Mitochondrial Analyses**

Mouse myocardial proteins were size-separated by 10% SDS-PAGE, transferred to PVDF membranes, and blocked with phosphate-buffered saline (PBS) and 0.1% Tween-20 (PBS-T) plus 5% nonfat dry milk before being incubated with primary antibodies (Abcam mouse polyclonal anti-Mfn1 [1:1000 dilution], mouse mononuclear anti-Mfn2 [1:1000 dilution], Cell Signaling Rabbit polyclonal anti-SERCA [1:1000], Abcam mouse monoclonal anti-R YR [1:1000 dilution], Abcam mouse monoclonal anti-NCX1 [1:500], Santa Cruz goat polyclonal anti-PLN [1:1000], and Sigma mouse monoclonal anti-A-TUBULIN [1:5000 dilution]). Secondary antibody was goat anti-rabbit immunoglobulin G (IgG), or goat anti-rabbit immunoglobulin G (IgG; 1:5000 dilution, Cell Signaling, Danvers, MA) or donkey anti-goat immunoglobulin G (IgG; 1:2000 dilution, Santa Cruz, CA) visualized using the ECL-Plus chemiluminescence reagent (GE Healthcare). Mitochondrial-associated membranes (MAMs) were obtained as described previously.³⁰

**Measurement of [Ca²⁺]ᵢ and [Ca²⁺]cm in Isolated Cardiomyocytes**

LV cardiac myocytes were isolated by enzymatic digestion, and recordings of [Ca²⁺]ᵢ together with [Ca²⁺]cm were performed using a patch-clamp–based approach as described previously.²²,²³ Briefly, myocytes were loaded with cell-permeable rhod-2 AM (for [Ca²⁺]ᵢ) and then patch-clamped and dialyzed with a pipette solution that contained a K⁺-glutamate based pipette solution (for composition, see Online Data Supplement) containing cell-impermeable indo-1 salt to monitor [Ca²⁺]ᵢ. Myocytes were held at ~70 mV in voltage-clamp mode and then depolarized to +10 mV for 100 ms at 0.5 Hz. After 60 seconds, isoproterenol (30 mmol/L) was added, and after 180 seconds, stimulation frequency was increased to 5 Hz (with depolarizing steps shortened to 50 ms). After 3 minutes, isoproterenol was washed out and stimulation frequency was set back to 0.5 Hz.

**Fluorescence Recordings in Field-Stimulated Cardiomyocytes**

Isolated cardiomyocytes were paced by electric field stimulation using a customized IonOptix system as described previously.²⁶ The autofluorescences of NAD(P)H/NAD(P)⁺ and FADH₂/FAD were determined by alternately exciting cells at wave lengths (λ(exc)) of 340 and 485 nm and collecting emission at λ(em) of 450 and 525 nm for NAD(P)H/FAD⁺, respectively. Calibration was performed with FCCP (5 µmol/L) and cyanide (4 mmol/L).²⁶ To detect mitochondrial superoxide (O₂⁻), myocytes were loaded with MitoSOX (3.3 µmol/L) for 30 minutes at 37°C (λ(exc)=380 nm, λ(em)=580 nm). As a positive control, antimycin A (150 µmol/L) was used. [Ca²⁺]ᵢ was measured by incubating cells with indo-1 AM (5 µmol/L) for 20 minutes at 25°C (λ(exc)=340 nm, λ(em)=405/485 nm). To monitor ∆Ψᵢ, myocytes were incubated with TMRM for 10 minutes at 25°C, and fluorescence intensity (λ(exc)=540 nm, λ(em)=605 nm) was determined before and after application of FCCP (4 µmol/L) and oligomycin (1.26 µmol/L) to completely dissipate ∆Ψᵢ.

**Mitochondrial Swelling Assay**

Mitochondrial swelling assays were performed as reported previously.³²

**L-Type Ca²⁺ Channel Electrophysiological Recordings**

Experiments were performed as described previously³³ and as outlined in more detail in the Online Data Supplement.

**Statistical Analysis**

Data are reported as mean±SEM. Probability values <0.05 were considered significant. For comparisons between 2 groups, an unpaired t test was performed unless indicated otherwise.

**Results**

Cardiomyocyte SR Ca²⁺ Release Is Increased in Mitofusin-Deficient Drosophila Heart Tubes

An essential role for mitofusin proteins in cardiac function was recently described in a *Drosophila* model wherein the single fruit fly mitofusin ortholog MARF was suppressed specifically in the heart tube.¹⁰ Loss of cardiomyocyte mitofusin/MARF produced mitochondrial fragmentation and dilated cardiomyopathy. Human and experimental cardiomyopathies induce characteristic alterations of cytosolic Ca²⁺ homeostasis.³⁴ Because the consequences of mitofusin/MARF insufficiency on cardiomyocyte Ca²⁺ handling were unknown, we measured [Ca²⁺]ᵢ in *Drosophila* heart tubes using an improved genetically encoded Ca²⁺ indicator, GCaMP3.0.³⁵ Ca²⁺ measurements were performed in intact, spontaneously contracting heart tubes (Figure 1A, left). Cardiomyocyte-specific RyR deficiency in *Drosophila*, studied as a positive control for altered SR Ca²⁺ export. Change in fluorescence was measured over a 150×100 pixel area centered over the conical region of the heart tube. Images were acquired at ~150 frames per second using a Photometrics Evolve EMCCD camera. Image analysis was performed with Nikon NIS elements 3.0 Advanced software.

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for the Ca\(^{2+}\) studies; the characteristic heart tube remodeling and contractile depression were unchanged (Figure 1D). Together, these findings indicate that cardiomyocyte-specific suppression of the fruit fly mitofusin ortholog MARF atypically alters SR Ca\(^{2+}\) handling. 

Postnatal Cardiac Ablation of Murine Mfn1 and Mfn2

Because *Drosophila* MARF is the single ortholog of both mammalian mitofusins, Mfn1 and Mfn2, the closest murine genetic counterpart to cardiomyocyte-specific MARF sup-
pression is combined cardiomyocyte-specific ablation of mfn1 and mfn2. We recently reported that cardiac Mfn1 and Mfn2 double knockout mice die at embryonic day 10.5 and observed that conditional combined Mfn1 and Mfn2 ablation in adult mouse hearts induced a rapidly progressive lethal dilated cardiomyopathy with mitochondrial fragmentation.11 Within a week of tamoxifen-induced cardiac double mfn gene ablation, the \([\mathrm{Ca}^{2+}]_c\) transients of Mfn1/Mfn2-deficient mouse cardiomyocytes suggested a trend toward increased amplitude.11 Thus, our studies in both the Drosophila and murine cardiac mitofusin insufficiency models implicated these mitochondrial fusion proteins in cardiomyocyte Ca\(^{2+}\) signaling. Defining a specific mechanism whereby one or the other Mfn protein affected Ca\(^{2+}\) handling required selective ablation of mfn1 or mfn2 in the mouse heart to uncover specific effects of either mitofusin on cardiomyocyte Ca\(^{2+}\) handling without inducing mitochondrial fragmentation (because they are redundant for this function\(^{3-5}\)). Accordingly, we crossed mfn1 and mfn2 floxed allele mice\(^4\) onto a MYH6 directed nuclear-localized (“turbo”) Cre transgene\(^{24}\) that induces gene recombination in cardiomyocytes beginning in the early postnatal period (Figure 2A through 2C) without confounding cardiotoxicity.\(^{18,36}\)

Mfn1 and Mfn2 cardiac-deficient mice were born at expected mendelian ratios (Online Table I). Mfn1 and Mfn2 immunoreactivities were decreased by \(\approx 80\%\) in the respective cardiac knockout mice, without compensatory upregulation of the non-targeted mitofusin (Figure 2A and 2B). LV dimension and ejection performance and the contractile responses at baseline and after \(\beta_1\)-adrenergic stimulation with dobutamine were normal in 6-week-old cardiac Mfn1-knockout (KO) (Figure 2D and 2F) and Mfn2-KO mice (Figure 2E and 2G). Mfn2-mediated mitochondrial fusion has been linked to protection from apoptosis,\(^{37}\) but there was no increase in TUNEL labeling or histological evidence of cardiomyocyte dropout in Mfn2-deficient hearts (Online Figure I).

**Mfn2-Containing Microdomains Modulate SR Ca\(^{2+}\) Handling**

Mfn2 tethers mitochondria to the ER in mouse embryonic fibroblasts,\(^{12}\) and physical tethering between ER and mitochondria is postulated to support microdomains through which Ca\(^{2+}\) transits between the organelles.\(^{14}\) Thus, disruption of these molecular tethers by ablation of Mfn2 may potentially also affect SR-mitochondrial Ca\(^{2+}\) signaling. To see if this paradigm applies to the mammalian heart, we measured cytosolic Ca\(^{2+}\) transients in ventricular cardiomyocytes from adult Mfn1- or Mfn2-deficient mice. Steady-state [Ca\(^{2+}\)]\(_c\) transients of Mfn1-KO cardiomyocytes (Fura-2; field-stimulated at 1 Hz) exhibited normal peak amplitudes and decay kinetics (Figure 3A). By comparison, cardiomyocyte Mfn2 deficiency produced slightly increased peak amplitudes of [Ca\(^{2+}\)]\(_c\) transients (Figure 3B). However, this increase was not associated with changes in the overall expression of Ca\(^{2+}\) transporting proteins in Mfn2-KO hearts (Figure 3C).

Increased [Ca\(^{2+}\)]\(_c\), after electric field stimulation is the consequence of Ca\(^{2+}\) influx through sarcoplasmic L-type Ca\(^{2+}\) channels and Ca\(^{2+}\) release through SR RyRs.\(^{38}\) In whole-cell voltage-clamp recordings, L-type Ca\(^{2+}\) channel current (\(I_{\mathrm{Ca,L}}\)) density was not different between Mfn2-KO and control myocytes (Figure 3D). An alternate explanation for the abnormally increased cardiomyocyte [Ca\(^{2+}\)]\(_c\) amplitude in Mfn2-KO cardiomyocytes is increased SR Ca\(^{2+}\) load or decreased locally activated mitochondrial Ca\(^{2+}\) clearance. In fact, direct activation of RyRs by caffeine resulted in larger [Ca\(^{2+}\)]\(_c\) increases in Mfn2-KO myocytes, whereas no difference was observed between Mfn1-KO and control myocytes (Figure 3E and 3F). The time constants (\(\tau\) values) for Ca\(^{2+}\) decay after caffeine and after steady-state Ca\(^{2+}\) transients were unchanged in Mfn1- and Mfn2-deficient myocytes, compared with their respective controls (Figure 3A and 3B and data not shown), indicating similar NCX and SR Ca\(^{2+}\) ATPase activities, respectively.

**Cardiac Mfn2 Deficiency Impairs Mitochondrial-SR Tethering**

These data raised the possibility that deletion of cardiomyocyte Mfn2 alters mitochondrial-SR Ca\(^{2+}\) crosstalk rather than primarily affecting cytosolic Ca\(^{2+}\) handling. To test this, we isolated cardiac mitochondria and examined the MAMs (the shared ER/SR and mitochondrial membranes) for differences in SR protein content driven by Mfn ablation. Indeed, the content of SR-associated RyR2 protein in cardiac MAMs was strikingly depressed by Mfn2 ablation compared with their respective controls (Figure 3G), despite unchanged overall cardiac RyR2 content (Figure 3C). Thus, the physical coupling to the mitochondria of the SR subdomains hosting RyR2 (terminal cisternae or junctional SR [jSR]) became weaker on Mfn2 ablation. Also in Mfn1-KO MAMs, RyR content appeared to be slightly diminished; however, the effect was clearly less pronounced than in Mfn2-KO hearts (Figure 3G), suggesting an indirect effect of Mfn1 ablation on SR-mitochondrial interactions. Alternatively, one could speculate that homotypic (Mfn2-Mfn2) SR-mitochondrial tether formation cannot compensate 100% for the loss of the heterotypic (SR Mfn2–mitochondrial Mfn1) tethers in Mfn1-KO hearts.

To further investigate mitochondrial morphology and in particular, SR-mitochondrial interaction, we analyzed transmission electron micrographs of Mfn1-KO and Mfn2-KO and their respective control hearts (Figure 4). In agreement with the data of Papanicolaou et al.,\(^{18}\) the mitochondrial area was increased in Mfn2-KO hearts (Figure 4A and 4C, top) without an increase in the mitochondrial area density (relative to sarcoplasmic area; data not shown). Likewise, the major axis of a fitted ellipse was longer in the Mfn2-KO mitochondria \((1.35\pm0.025 \, \text{m\mu} \text{m} \text{versus} \ 1.09\pm0.01 \, \text{m\mu} \text{m}; n=978 \text{and} 1317 \text{in} \ \text{Mfn2-KO and control hearts, respectively}; \ P<0.05\). The transversal side length of mitochondria in close association with jSR was unchanged with Mfn2 ablation (Figure 4C, bottom left), but the transversal side segment forming an interface with jSR was reduced by \(\approx 30\%\) in Mfn2-KO versus control hearts (Figure 4C, bottom right). Furthermore, there was a trend toward an increase in the mean distance between jSR and the outer mitochondrial membrane in Mfn2-KO versus control hearts (by \(\approx 17\%\); \(P=0.07\); data not shown). In contrast, none of these parameters were altered in Mfn1-KO hearts compared with their respective controls (Figure 4B).
Taken together, loss of Mfn2, but not Mfn1, substantially diminishes the physical interaction between SR and mitochondria in cardiac myocytes.

Ablation of Cardiac Mfn2, But Not Mfn1, Disrupts the Mitochondrial Bioenergetic Response

To test whether the alterations of SR-mitochondrial interaction affect mitochondrial Ca\(^{2+}\) uptake under physiological conditions, we applied a patch-clamp–based approach that we previously established in guinea pig cardiac myocytes.\(^{26,31,39}\) Intact murine cardiac myocytes were loaded with the cell-permeable Ca\(^{2+}\)-indicator rhod-2 AM, which localizes primarily to mitochondria. To eliminate contaminating signals from cytosol-located rhod-2 AM, myocytes were whole-cell patch-clamped and dialyzed with a pipette solu-
tion that did not contain rhod-2, but (membrane-impermeable) indo-1 salt. With this technique, we monitored \([\text{Ca}^{2+}]_m\) (by rhod-2) together with \([\text{Ca}^{2+}]_c\) (by indo-1) in "working" cardiac myocytes that were voltage-clamped and depolarized to \(-10\) mV at 0.5 Hz, inducing steady-state \(\text{Ca}^{2+}\) transients. To impose a near physiological workload, isoproterenol was applied and the rate of depolarization was increased to 5 Hz for 3 minutes (Figure 5B and 5D). In agreement with our previous results,31,39 rapid mitochondrial \(\text{Ca}^{2+}\) transients were observed during cytosolic \(\text{Ca}^{2+}\) transients, with diastolic accumulation of \([\text{Ca}^{2+}]_m\) during \(\beta\)-adrenergic stimulation and an increase in pacing rate (Figure 5A through 5D). To rule out that beat-to-beat \([\text{Ca}^{2+}]_m\) transients reported by rhod-2 derived from cytosolic traces of the dye, we performed control experiments with an inhibitor of the MCU (1 \(\mu\)mol/L of Ru360 in the pipette solution; Online Figure II). Ru360 reduced the amplitude of \([\text{Ca}^{2+}]_m\), but not \([\text{Ca}^{2+}]_c\) transients and blunted the diastolic accumulation of \([\text{Ca}^{2+}]_m\), confirming specific localization of rhod-2 to mitochondria31,39 (Online Figure II).

Whereas in Mfn1-deficient myocytes, mitochondrial \(\text{Ca}^{2+}\) uptake was unchanged compared with control myocytes...
Figure 4. Cardiomyocyte mitochondrial-SR architecture is altered by Mfn2 ablation. A, Transmission electron micrographs of longitudinal sections of myocardium derived from control (ctrl) (top) and cardiac Mfn2-null (Mfn2-KO) mice (bottom). Lower-magnification overview images on the left show the overall mitochondrial distribution and morphology. Fivefold-higher magnification of the framed areas are shown on the right with arrows pointing to SR-mitochondrial associations. B and C, Cumulative analysis of mean perimeter (top, left) and area of mitochondria (top, right) and of the transverse side length (bottom, left) and contact length with jSR (bottom, right, respectively) in Mfn1-KO (B) and Mfn2-KO (C) mice compared with their respective controls. Mfn1-KO (B): n=8 and 6 cellular areas analyzed from 2 different hearts each for Mfn1-KO and control, respectively; each cellular area represents the means/sum of 117 to 377 and 144 to 299 individual mitochondria. Mfn2-KO (C): n=8 and 7 cellular areas from 4 different hearts each for Mfn2-KO and control, respectively; each cellular area represents the means/sum of 44 to 173 and 65 to 304 individual mitochondria. *P<0.05 (Mann-Whitney rank sum test).
Figure 5. Impaired mitochondrial Ca\(^{2+}\) accumulation and bioenergetic feedback response in Mfn2-deficient myocytes. Experiments were performed on intact cardiac myocytes with acute isoproterenol and pacing stress (see Online Figure IV); Mfn1-KO on left, Mfn2-KO on right. A and C, Averaged original traces of [Ca\(^{2+}\)]\(_{c}\) (top) and [Ca\(^{2+}\)]\(_{m}\) transients (middle) in WT and Mfn-KO myocytes after isoproterenol (30 nM) for 1 minute at 0.5 Hz. Bottom panels show dynamic changes of [Ca\(^{2+}\)]\(_{m}\) plotted against [Ca\(^{2+}\)]\(_{c}\) in the same cell in the presence of isoproterenol for 1 minute at 0.5 Hz (Mfn1: n=4 control, n=7 KO; Mfn2: n=14 control, n=12 KO). B and D, Time-dependent changes in diastolic [Ca\(^{2+}\)]\(_{m}\) with pacing and isoproterenol stress. Inset in D shows change of diastolic [Ca\(^{2+}\)]\(_{m}\) in the first 2 minutes after application of isoproterenol. E and F, Autofluorescence of NAD(P)H (top), FAD (middle), and the ratio of NAD(P)H/FAD (bottom; Mfn1-KO, n=17; control, n=7; Mfn2-KO, n=24; control, n=15). *P<0.05 and **P<0.01 WT versus KO, respectively (ANOVA for repeated measures).

(Figure 5A and 5B), the dynamic relationship between [Ca\(^{2+}\)]\(_{m}\) and [Ca\(^{2+}\)]\(_{c}\) was shifted toward lower efficiency of mitochondrial Ca\(^{2+}\) uptake in Mfn2-deficient myocytes (Figure 5C). At 0.5 Hz, mitochondrial Ca\(^{2+}\) accumulation in response to \(\beta\)-adrenergic stimulation was completely blunted in Mfn2-KO myocytes compared with controls (Figure 5D and inset). At 5 Hz and maintained presence of isoproterenol, mitochondrial Ca\(^{2+}\) accumulation remained decreased in Mfn2-KO myocytes compared with controls but equilibrated in the course of this stimulation to similar levels (Figure 5D).

To further elucidate whether the differences in mitochondrial Ca\(^{2+}\) accumulation in Mfn2-KO and wild-type (WT) myocytes were related to differences in mitochondrial Ca\(^{2+}\) uptake and/or decay kinetics, we determined the time-to-peak (TTP) and time to 50% decay (RT50) of cytosolic and mitochondrial Ca\(^{2+}\) uptake, respectively (ANOVA for repeated measures).
making differences in Ca\(^{2+}\)-induced Krebs cycle activation the most likely explanation for net NAD(P)H and FAD oxidation. As reduced NADPH is required for elimination of ROS from the matrix, mitochondrial \(\text{O}_2^-\) production was temporarily increased by isoprotrenol challenge of Mfn2-KO cardiomyocytes (Figure 6A).

The observed bioenergetic and redox mismatches in Mfn2-deficient cardiomyocytes were not related to an intrinsic defect in mitochondrial Ca\(^{2+}\) uptake because both the mitochondrial membrane potential (\(\Delta\Psi_m\)) as the driving force for mitochondrial Ca\(^{2+}\) uptake (Figure 6B) and Ca\(^{2+}\) sensitivity of mitochondrial swelling (Figure 6C) were similar. These results suggest that Mfn2 plays a role in the regulation of SR-mitochondrial calcium crosstalk but not intrinsic mitochondrial sensitivity to Ca\(^{2+}\). Furthermore, similar \(\Delta\Psi_m\) values (Figure 6B) and similar cardomyocyte performance at elevated work (Online Figure IV) suggest that Mfn2 deficiency had no direct impact on respiratory chain function or ATP production.

**Discussion**

In these studies of genetically manipulated *Drosophila* heart tubes and mouse hearts, we provide evidence that Mfn2 bridges cardiomyocyte SR and mitochondria, thus facilitating interorganelle Ca\(^{2+}\) crosstalk that regulates the immediate bioenergetic response to increased cardiac work. This function of Mfn2 is not shared by Mfn1 and appears independent of effects on mitochondrial fusion.\(^4\)\(^5\)\(^11\)

The concentration and contextual release of Ca\(^{2+}\), a ubiquitous signaling molecule, are linked to a variety of essential physiological as well as many pathological cellular processes. Intracellular Ca\(^{2+}\) is heavily sequestered within storage organelles, largely SR/ER, thus maintaining relatively low levels of \([\text{Ca}^{2+}]_i\). In cardiac myocytes, \([\text{Ca}^{2+}]_i\) oscillates from a baseline (diastolic) concentration of \(\sim 100 \text{ nmol/L}\) to a peak level of \(\sim 1 \text{ mmol/L}\) with each cardiac systole. A small fraction of this Ca\(^{2+}\) enters through sarclolemal L-type Ca\(^{2+}\) channels, but the majority is released from SR via RyRs and taken up again via SR Ca\(^{2+}\) ATPase.\(^7\)\(^8\)

Mitochondria are essential to cardiac SR Ca\(^{2+}\) cycling as the source of ATP that fuels various ion pumps and myosin ATPase. Accordingly, SR and mitochondria are organized throughout cardiac myocytes in an intricate and intimate physical association. Spatial colocalization of mitochondria with SR facilitates compartmentalization and privileged transport of ATP, and potentially also for Ca\(^{2+}\). In mitochondria, Ca\(^{2+}\) plays a key role in matching energy supply and demand by stimulating rate-limiting enzymes of the Krebs cycle, the main producer of NADH.\(^6\)\(^7\) Because regeneration of antioxidative NADPH is coupled to the Krebs cycle, mitochondrial Ca\(^{2+}\) uptake also plays an important role in preventing excess formation of toxic ROS.\(^31\) However, the low Ca\(^{2+}\) affinity of the primary mitochondrial Ca\(^{2+}\) uptake mechanism (ie, the MCU; \(EC_{50} \approx 10 \text{ mmol/L}\)) limits the ability of mitochondria to import Ca\(^{2+}\) from the cytosol, where Ca\(^{2+}\) levels are normally much lower.\(^14\) To resolve this apparent paradox, Rizzuto and Pozzan inferred the existence of Ca\(^{2+}\) signaling “hot-spots,” or microdomains, between ER and mitochondria.\(^42\)\(^43\) Indirect evidence has previously pointed to a role for localized SR-mitochondrial Ca\(^{2+}\) signaling in cardiac myocytes\(^44\)\(^46\) and (although the molecular mediator was unknown) SR-mitochondrial bridging has been observed in heart and striated muscle.\(^47\)\(^48\) Csorbas et al defined structural and functional mitochondrial-ER connections,\(^13\)\(^49\) and de Brito and Scorrano established a molecular mechanism for physical tethering of mitochondria to ER by Mfn2 in embryonic fibroblasts.\(^12\)

Our results demonstrate that the concept proposed by de Brito and Scorrano of Mfn2 as a molecular tether between ER and mitochondria\(^12\) is applicable to the distinct subcellular structure and organ physiology of the heart, in which the SR is the main Ca\(^{2+}\) store. The heart is the most mitochondria-rich organ and requires high and quickly modifiable rates of ATP generation to maintain organ pumping function under different work loads. Cardiac EC coupling is dependent on constant cyclic SR Ca\(^{2+}\) release and reuptake.\(^38\) Disturbances in either mitochondrial metabolism or SR Ca\(^{2+}\) cycling induce cardiac dysfunction and can cause heart disease.\(^50\)\(^51\)
For these reasons, we postulated that the unique sensitivity of the heart to reticular and mitochondrial dysfunction would make it an ideal experimental platform in which the molecular determinants of mitochondrial-SR crosstalk could be functionally interrogated. Our findings reveal that SR-mitochondrial tethering by Mfn2 is essential for mitochondrial sensing of stress-induced SR Ca\textsuperscript{2+} release, linking mitochondrial ATP production to SR Ca\textsuperscript{2+} cycling in a rapid-response system that prevents bioenergetic lag when cardiac work is acutely increased. However, we cannot exclude the possibility that also other proteins—such as PACS-2 or IP\textsubscript{3}-receptors interacting with VDAC in the ER-mitochondrial interaction of other cell types\textsuperscript{19,20}—may contribute to SR-mitochondrial crosstalk in cardiac myocytes.

Although our findings confirm and expand on those of de Brito and Scorrano,\textsuperscript{12} they contrast with those of Papanicolau et al.\textsuperscript{18} who recently reported that cardiac-specific ablation of Mfn2 in mice does not alter the close associations between SR and mitochondria (based on distance measurements) or affect cardiomyocyte Ca\textsuperscript{2+} signaling, and that loss of Mfn2 protected against ischemia-induced opening of the mitochondrial permeability transition pore.\textsuperscript{18} There are important differences between our studies and those of Papanicolau et al. First, the Cre line used in the previous study is well known to express Cre at sufficiently high levels in the mouse embryo and adult that it induces cardiotoxicity in older mice.\textsuperscript{22} The nuclear-directed Cre line used in our studies induces recombination only after birth (vide supra) and has not been reported to induce any toxicity.\textsuperscript{22} Indeed, since the same Cre excision strategy was used in our parallel experiments for both Mfn1 and Mfn2, direct Cre effects have not contributed to our findings.

Second, for the critical issue of whether ablation of Mfn2 from cardiac myocytes alters the close associations between SR and mitochondria, that is, whether Mfn2 actually tethers the two organelles as described by de Brito and Scorrano, differences in the morphometric analysis may explain Papanicolau’s negative findings. Their analysis was restricted to measurements of the distance between the center of T-tubules and mitochondria, which was in the range of 150 nm and not different between Mfn2-KO and control animals.\textsuperscript{18} In contrast, we analyzed the distance between the jSR and mitochondria, which is in the range of only 15 nm and a more direct parameter for SR-mitochondrial tethering than the distance from the T-tubule center to mitochondria. In these measurements, we observed a trend toward a widening of the gap between SR and mitochondria in Mfn2-KO compared with control mice (P=0.07). Moreover, another important parameter for the functional interaction between SR and mitochondria is the actual contact length of mitochondria with the jSR, which was decreased by \textapprox 30\% in Mfn2-KO but not Mfn1-KO hearts in our study but was not analyzed in the study of Papanicolau et al.\textsuperscript{18} As the SR-mitochondrial physical coupling is predicted to be established by more than one tether species,\textsuperscript{52} eliminating only one of these tethers (Mfn2) may decrease interface formation with or without a change in the gap distance of the remaining associations. These ultrastructural differences were corroborated by the substantially decreased content of SR-specific RyRs in MAMs isolated from Mfn2-KO (but not Mfn1-KO) hearts, demonstrating a mechanically more vulnerable coupling between SR and mitochondria.

The kinetics of mitochondrial Ca\textsuperscript{2+} uptake are still subject to debate,\textsuperscript{7,15–17} which is primarily related to the low affinity of the MCU for Ca\textsuperscript{2+} and differential results yielded by the use of different techniques. While some observers have described beat-to-beat cardiomyocyte mitochondrial Ca\textsuperscript{2+} transients, others have observed only slow Ca\textsuperscript{2+} accumulation during increases of the amplitudes and/or rate of cytosolic Ca\textsuperscript{2+} transients.\textsuperscript{7,15–17} The existence of RyR-derived high Ca\textsuperscript{2+} microdomains facilitating Ca\textsuperscript{2+} delivery to mitochondria was inferred from data in permeabilized H9c2 myotubes\textsuperscript{44} and cardiomyocytes.\textsuperscript{55} We have also previously used computational modeling and electrophysiological studies to support the existence of Ca\textsuperscript{2+} microdomains between closely associated mitochondria and SR, within which “hot spots” of Ca\textsuperscript{2+} can accumulate at sufficiently high concentrations (because of limited diffusion) to be imported by the low-affinity MCU.\textsuperscript{26,31,39} We demonstrate that Mfn2 is a critical structural component to these microdomains and to mitochondrial uptake of Ca\textsuperscript{2+} release from juxtaposed SR. The weakened associations of jSR and mitochondria in Mfn2-ablated cardiomyocytes lead to increased pacing- as well as caffeine-triggered [Ca\textsuperscript{2+}]\textsuperscript{i} signal amplitudes without changes in the expression levels of SR Ca\textsuperscript{2+} handling proteins, or in the activity of high-affinity Ca\textsuperscript{2+} extrusion mechanisms (SERCA, NCX). Thus, these increased [Ca\textsuperscript{2+}]\textsuperscript{i} signals were not likely to be due to enhanced SR Ca\textsuperscript{2+} accumulation or decreased sarcolemmal Ca\textsuperscript{2+} extrusion but rather to the decreased local mitochondrial Ca\textsuperscript{2+} clearance.

The primary physiological role of mitochondrial Ca\textsuperscript{2+} uptake is the stimulation of rate-limiting enzymes of the Krebs cycle to adapt energy supply and demand.\textsuperscript{6,7} In fact, the redox states of NAD(P)H/NAD(P)\textsuperscript{+} and FADH\textsubscript{2}/FAD\textsuperscript{+}, and the ratio of NAD(P)H/FAD\textsuperscript{+} (an index of the balance between Krebs cycle-induced reduction versus oxidation through the respiratory chain) were more oxidized during \textbeta-adrernergic stimulation in Mfn2-KO than in control cardiomyocytes. Furthermore, we recently discovered that mitochondrial Ca\textsuperscript{2+} uptake during \textbeta-adrernergic stimulation prevents mitochondrial ROS formation by buffering the NADPH-dependent antioxidative capacity.\textsuperscript{31} Indeed, mitochondrial O\textsubscript{2}− increased slightly within the first minute of isoproterenol, which may be related to oxidation of NADPH secondary to a mismatch of ADP-induced oxidation and Ca\textsuperscript{2+}-induced reduction of NAD(P)H.\textsuperscript{31} Suppression of the Drosophila mitofusin ortholog MARF induced dilated cardiomyopathy and cardiomyocyte mitochondrial fragmentation; the former was improved by cardiac expression of superoxide dismutase, whereas the latter was not improved.\textsuperscript{10} This observation and the differences between single Mfn1 and Mfn2 cardiac knockout mice described here and double Mfn1/Mfn2 cardiac knockout mice recently reported further emphasize the different roles of mitofusins as mediators of mitochondrial fusion and as modulators of SR-mitochondria Ca\textsuperscript{2+} transport/bioenergetics/ROS production. Our data suggest that the defects in the spatial organization of mitochondria and SR that depress mitochondrial...
Ca\textsuperscript{2+} uptake may also contribute to myocardial oxidative stress.

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Disclosures

None.

References

What Is Known?

- Mitofusin 2 tethers endoplasmic reticulum to mitochondria in embryonic fibroblasts.
- Mitochondrial Ca\(^{2+}\) uptake is important for matching energy supply and demand and control mitochondrial emission of reactive oxygen species.
- In cardiac myocytes, mechanisms that tether sarcoplasmic reticulum (SR) to the mitochondria are unclear, and the role of calcium crosstalk remains unknown.

What New Information Does This Article Contribute?

- In adult cardiac myocytes, mitofusin 2 but not mitofusin 1 tethers the SR to mitochondria. Tethering creates Ca\(^{2+}\) microdomains between the organelles that affects mitochondrial Ca\(^{2+}\) handling.
- SR-mitochondrial Ca\(^{2+}\) crosstalk via microdomains directs mitochondrial metabolism to increased contractile demand, avoiding bioenergetic mismatch and oxidative stress.

Novelty and Significance

The heart consumes large amounts of energy that must be produced by mitochondria. For efficient energy regeneration, mitochondria take up Ca\(^{2+}\) that stimulates key enzymes of the Krebs cycle to increase the production of substrates of the electron transport chain. Since the mitochondrial Ca\(^{2+}\) uniporter has a relatively low affinity for Ca\(^{2+}\), the kinetics of mitochondrial Ca\(^{2+}\) uptake are currently unclear. Here, we identify mitofusin 2 as a protein that tethers mitochondria to the SR, providing a close spatial interaction between both organelles that accounts for efficient mitochondrial Ca\(^{2+}\) uptake during changes of cardiac work load. At the same time, mitochondrial Ca\(^{2+}\) uptake shapes cytosolic Ca\(^{2+}\) signals. This function of mitofusin 2 is not related to its role in mitochondrial fusion and is not shared by its closely related homologue, mitofusin 1. Disruption of SR-mitochondrial Ca\(^{2+}\) crosstalk hampers energy supply and demand and may produce oxidative stress. These results demonstrate an essential physiological function of SR-mitochondrial tethering in controlling SR-to-mitochondria calcium delivery and matching mitochondrial bioenergetics to acute cardiac demand.
Mitofusin 2-Containing Mitochondrial-Reticular Microdomains Direct Rapid Cardiomyocyte Bioenergetic Responses Via Interorganelle Ca\textsuperscript{2+} Crosstalk

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Supplemental Information

SUPPLEMENTAL METHODS

Ultrastructure studies
From mice killed via cervical dislocation the heart was rapidly removed and small (~1-2 mm³) pieces of the left ventricular free wall were immersion-fixed in Karnovsky’s fixative. The fixed samples were stained en block with partially reduced osmium tetroxide (0.8% potassium-ferrocyanide and 2% osmium tetroxide in 0.1 M sodium cacodylate buffer overnight at 4°C) and in turn with 1% uranyl acetate (1hr at 4°C). Stained samples were dehydrated on an acetone dilution series and embedded in Spurr’s resin according to manufacturer’s (Electron Microscopy Sciences) instructions. 60-100 nm thin sections were cut from the embedded specimens, mounted on electron microscopy grids and examined using a FEI Tecnai 12 TEM equipped with a phosphor plate (Advanced Microscopy Techniques, AMT) and Hamamatsu Orca 8Mpx digital camera.

Morphometric analysis of mitochondria and the SR-mitochondrial associations was carried out on longitudinal sections using MacBiophotonics’ Image J software. For the generic characterization of mitochondrial morphology and abundance, a mask was drawn over the largest possible sarcoplasmic area (without subsarcolemmal and perinuclear regions) in a cardiomyocyte section, and under this mask for each mitochondrial cross sections the area, perimeter and the major axis of the fitted ellipse were determined. The percent of the sarcoplasmic area covered by mitochondria was used as the 2D equivalent of mitochondrial volume density. The number of individual mitochondrial cross sections per 10 µm² was also determined that together with the mitochondrial density reflected on the fusion/fragmentation states.

For the morphometric analysis of SR-mitochondrial associations a comprehensive protocol is yet to be established. Most of the published TEM analyses focus on the distance between the interfacing mitochondrial and SR membranes ¹ or T-tubule centers ². However, as the platforms for local functional crosstalk, besides their tightness the extent of these associations is just as important in a comparative analysis. We have introduced a protocol in this regard for the analysis of ER-mitochondrial associations in non-muscle cells in 2006 ³ that we adopted now for the quantification of SR-mitochondrial associations in the cardiac muscle as follows. Areas where the junctional SR (jSR) was <50 nm away from the outer mitochondrial membrane were accounted as jSR-mitochondrial interfaces. Since the SR-derived Ca²⁺ signals that locally propagate to the mitochondria in the ventricular muscle are generated mainly in the T-tubule SR junctions (dyads), the analysis was restricted to the intermyofibrillar mitochondria (excluding the distinct sub-sarcolemmal and perinuclear mitochondrial population). This restriction also applied to the generic mitochondrial parameters. The dyad-forming SR regions are the terminal cisternae (jSR) that are localized to the Z disks and so they mostly associate with the transversal side of the mitochondrion, while the longitudinal sides are enmeshed by the network SR⁴, ⁵. Hence, to reference the extent of jSR-mitochondria associations we chose to quantify the length of the mitochondrial interface as the fraction of the participating mitochondrial transversal side (100 % meaning a transversal side fully covered by jSR). Importantly, the length of these transversal sides was not different between Mfn1-/- and Mfn2-/- and their respective control samples (Fig. 4b and 4c). For the SR-mitochondrial gap distance for each associations 3-5 individual distance readings (evenly distributed over the interface length) were averaged. For the cumulative analysis, each contributing mitochondrion was represented by a single distance
number. If a mitochondrion (transversal side) had more than one jSR association, a weighted average (based on surface representation) was created from the distance values of these associations.

**L-type Ca\(^{2+}\) channel electrophysiological recordings**

Whole-cell recordings were obtained from LV myocytes within 12h of isolation at room temperature. Experiments were performed using a Dagan 3900A (Dagan Corporation, Minneapolis, MN, USA) patch clamp amplifier interfaced to a microcomputer with a Digidata 1332 analog/digital interface and the pCLAMP9 software package (Molecular Devices). Data were filtered at 5 kHz before storage. For recordings of whole-cell Ca\(^{2+}\) currents (\(I_{Ca,L}\)), pipettes contained (in mM): CsCl 135; EGTA 10; HEPES10; glucose 5.5; MgATP 3 and TrisGTP 0.4 (pH 7.2; 310 mOsm). The bath solution contained (in mmol/L): TEACl 140; KCl 4; MgCl\(_2\) 2; CaCl\(_2\) 2; HEPES 10 and glucose 10 (pH 7.4; 310mOsm). Currents were evoked in response to 400 ms voltage steps to test potentials between -40 and +50 mV from a holding potential (-40 mV) to inactivate voltage-gated Na\(^{+}\) currents. Data were compiled and analyzed using Clampfit (Version 9.2, Molecular Devices) and Excel (Microsoft, Redmond, WA, USA). Integration of the capacitative transients, recorded during brief ±10 mV voltage steps from the holding potential (-70 mV), provided the whole-cell membrane capacitance (\(C_m\)). Leak currents were always <100 pA, and were not subtracted. Series resistances (<10 M\(\Omega\)) were routinely compensated electronically (>80%). Voltage errors resulting from the uncompensated series resistances were ≤8 mV and were not corrected. Peak \(I_{Ca,L}\) amplitudes were measured and normalized to whole-cell membrane capacitances (in the same cell) and current densities (in pA/pF) were compared.

**Patch clamp solutions**

Solutions used in patch-clamp and field-stimulation experiments (Figure 5; Supplemental Figures III, IV):

- **Normal Tyrode´s** ( perfusion) solution containing (in mmol/L): NaCl 130, KCl 5, MgCl\(_2\) 1, CaCl\(_2\) 2, Na-HEPES 10, glucose 10, Na-pyruvate 2 and ascorbic acid 0.3, pH 7.4.
- **Pipette solution** (in mmol/L): K-glutamate 130, KCl 19, MgCl\(_2\) 0.5, Na-HEPES 15, Mg-ATP 5, pH 7.2.
Online Figure I.

**TUNEL labeling of Mfn2 KO hearts.**

**A.** Representative TUNEL labeling fluorescent micrographs of control and Mfn2 KO cardiac sections at 6-week and 16 weeks of age. Green: TUNEL positive labeling; Blue: DAPI nuclear counterstain. **B.** Quantitative TUNEL analysis ($n = 5$ per group).
Online Figure II:

**Ru360 inhibits mitochondrial, but not cytosolic Ca$^{2+}$ transients and accumulation.**

Recordings of [Ca$^{2+}$]$_c$ (A, C) and [Ca$^{2+}$]$_m$ (B, D), determined by indo-1 and rhod-2, respectively, in patch-clamped cardiac myocytes (from C57BL/6N mice) exposed to a protocol of 0.5 Hz voltage-clamp depolarizations and an increase to 5 Hz (grey areas) in the presence of isoproterenol. The pipette solution contained the MCU-inhibitor Ru360 (1 µM) or vehicle (Control), respectively. The data of the whole protocol (A, B) or averaged transients at 120 s (isoproterenol) are given. E and F, [Ca$^{2+}$]$_m$ plotted against [Ca$^{2+}$]$_c$ for single transients (at 5 Hz, 120 s; E) or as averaged values for the entire experiment (F) in the absence and presence of Ru360. *p<0.05 (ANOVA for repeated measures).
Online Figure III:

Cytosolic and mitochondrial Ca\(^{2+}\) upstroke and decay kinetics in Mfn2-KO and WT myocytes. A, Time-to-peak (TTP) of cytosolic and mitochondrial Ca\(^{2+}\) transients in all WT myocytes. B and C, TTP of [Ca\(^{2+}\)]\(_c\) (B) and [Ca\(^{2+}\)]\(_m\) (C) in WT and Mfn2-KO myocytes, respectively. D and E, Time to 50% decay of [Ca\(^{2+}\)]\(_c\) (D) and [Ca\(^{2+}\)]\(_m\) (E) in WT and Mfn2-KO myocytes, respectively. Data were obtained at steady-state 5 Hz stimulation in the presence of isoproterenol.
Online Figure IV.

**Cardiomyocyte bioenergetic stress response studies.**

Murine adult ventricular myocytes undergoing field-stimulation at 0.5 Hz were exposed to isoproterenol (30 nM) and stimulation frequency was subsequently increased to 5 Hz for 3 min.

A. Original trace of sarcomere shortening recordings with time course matched to the data sets in panels B, C and D, Figure 5 E and F. Fractional sarcomere shortening (B; FS), systolic and diastolic $[\text{Ca}^{2+}]_c$ (C) and amplitude of $[\text{Ca}^{2+}]_c$ transients (D) for Mfn2-/ (control and KO), respectively. n=30-50 cells for sarcomere shortening and 6-12 cells for $\text{Ca}^{2+}$ studies. *p<0.05.
Online Table I. Genotypes of Mfn1 and Mfn2 cardiac KO crosses.

<table>
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<th>Genotype</th>
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<th>Expected F/F x Cre</th>
<th>Observed F/F x Cre</th>
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<td>mfn1 x Myh6-Cre</td>
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<td>n=52</td>
<td>n=53</td>
</tr>
<tr>
<td>mfn2 x Myh6-Cre</td>
<td>n=298</td>
<td>n=149</td>
<td>n=138</td>
</tr>
</tbody>
</table>

Homozygous mfn floxed mice (mfn $^{lox/lox}$; F/F) were bred to mice homozygous for the same floxed mfn allele and heterozygous for either Myh6-CRE. There were no differences between observed and expected (Chi square test).

Supplemental References