Figure S1, related to Figure 1: Semi-logarithmic plot of $\text{Ca}^{2+}$ uptake rate versus $[\text{Ca}^{2+}]_c$ in liver and heart mitochondria.
Figure S2, related to Figure 3.
(A) Representative immunoblotting of MICU3, EMRE and Hsp70 in mice liver, heart and skeletal muscle mitochondrial lysates.
(B) Relative protein level of MICU3 and EMRE is displayed in the bar graph for each protein relative to the loading control Hsp70 (mean ± SEM, n=4, *p<0.05).
Figure S3.

(A) MCU, MICU1, MICU2 and Hsp70 (mitochondrial loading control) protein levels in HeLa cells upon MCU overexpression (MCU, blue) or knock-down (MCU-KD) in the upper panel, or after MICU1 downregulation (si1 and si2, red) in the lower panel.

(B) SOCE-induced [Ca\textsuperscript{2+}]c. Upper panels: time courses in Ctrl (black) or MCU overexpressing (blue) HeLa cells on left (thin line: [Ca\textsuperscript{2+}]c fura2, nM) and thick line: [Ca\textsuperscript{2+}]m inverse pericam, ipcam, F0/F) and in Ctrl or MICU1 silencing (si1 and si2) HeLa cells on right after addition of 0.2mM CaCl\textsubscript{2} (thin line: [Ca\textsuperscript{2+}]c RCaMP, F/F0 and thick line: [Ca\textsuperscript{2+}]m ratiometric pericam, rpcam, F0/F). Lower panels: for each time point [Ca\textsuperscript{2+}]m is plotted against [Ca\textsuperscript{2+}]c; traces represent individual cells.

(C) Fluorescence images of HeLa cells showing mitochondrial co-localization of TMRE and furaFF (upper) and OMP25-GFP and furaFF (lower). Scale bar: 10 µm.

(D) Time courses of [Ca\textsuperscript{2+}]m (furaFF) in permeabilized Ctrl and MCU overexpressing HeLa cells exposed to a 1.5 µM Ca\textsuperscript{2+} pulse in the absence and presence of RuRed.

(E) Representative traces of [Ca\textsuperscript{2+}]m recorded with furaFF in permeabilized Ctrl and MICU1 silenced HeLa cells.

(F) The means of the resting [Ca\textsuperscript{2+}]m are shown for the MCU overexpressing and MICU1 silenced cells (normalized to the respective Ctrl). *p<0.05.
Figure S4: MICUs to MCU and MICU1 to MICU2 protein ratios and mitochondrial Ca$^{2+}$ uptake, related to Figure 4.

(A) The protein ratios of MICU1 to MCU, MICU2 to MCU and MICU1 to MICU2 were calculated (mean ± SEM, n=3-4, *p<0.05 vs. AAV9-Luc) from Figure 4B and ratios were normalized to AAV9-Luc heart mitochondria.

(B) Representative time courses of the mitochondrial clearance of the [Ca$^{2+}$]$_c$ rise upon addition of a 3 µM CaCl$_2$ bolus (3Ca) in suspensions of AAV9-Luc (grey) and AAV9-MICU1 (pink) heart mitochondria, with and without RuRed (3 µM).

(C) Mitochondrial clearance of [Ca$^{2+}$]$_c$ elevations induced by 50 µM CaCl$_2$ addition.

(D) $\Delta\Psi_m$ measured with TMRM (used in de-quench mode thus the direction of polarization is downward).
Table S1: MCUb mRNA level in liver and heart tissues (mean ± SEM, n=4, *p<0.05 vs respective control).

|                | MCUb mRNA level          |
|----------------|--------------------------|
| Liver          | 7.31e-3 ± 4.03e-3        |
| Heart          | 9.25e-2 ± 3.57e-2*       |
| Heart AAV9-Luc | 1.54e-1 ± 5.49e-2        |
| Heart AAV9-MICU1| 1.61e-1 ± 3.32e-2       |
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Chemicals
Standard chemicals were purchased from Fisher Scientific or Sigma-Aldrich. Thapsigargin and CGP-37157 were from Enzo Life Sciences (Plymouth Meeting, PA). Fluorescent Ca²⁺-indicator dyes, and Pluronic F-127 were from Molecular Probes or Teflabs. Chelex 100 Sodium form was purchased from BioRad.

AAV and DNA constructs
AAV9-Luciferase was from Penn Vector Core (Philadelphia, PA) and AAV9-MICU1 was custom prepared by Vector Biolabs (Philadelphia, PA). FLAG tagged-MCU was described in (Patron et al., 2014).

Cell culture and transfection
HeLa cells were grown in DMEM, 10% FBS and 1% penicillin/streptomycin and were transiently transfected with siRNA or plasmid DNA as described earlier (Csordas et al., 2013). MICU1-KO HEKs were kindly provided by Dr. Vamsi Mootha and grown as previously described (Sancak et al., 2013). Transfection in HEKs cells was performed using Lipofectamine 3000 (Thermo Fisher).

Heart, skeletal muscle and liver mitochondria isolation
Mice were euthanized by cervical dislocation. Heart was rapidly excised and washed in cold isolation buffer containing 225mM Mannitol, 75mM Sucrose, 20mM Hepes and 0.1mM EGTA, at pH 7.4. Heart tissue was finely minced and incubated for 1min in the isolation buffer with 0.5% BSA and 3U/g of tissue of proteases (Sigma). Then, the cardiac tissue was homogenized with a glass Teflon potter homogenizer and the homogenate centrifuged at 500g/min at 4°C to remove nuclei and cellular debris. The supernatant was next centrifuged at 9,000g/10min/4°C and the mitochondrial pellet was resuspended in 225mM Mannitol, 75mM Sucrose, and 10mM Hepes at pH 7.4 and kept on ice. After liver harvesting, liver mitochondria were isolated by differential centrifugations. Briefly, liver was minced in a cold isolation buffer containing 250mM Sucrose, 10mM Tris/HCl, 0.1mM EGTA and 0.5% BSA, at pH 7.4 and homogenized in a glass Teflon potter. Cellular debris were removed by two consecutive centrifuges at 370g/10min/4°C. Supernatant was then centrifuged at 7,500g/10min/4°C and mitochondrial pellet washed once in isolation buffer without BSA before a new centrifugation at 7,500g/10min/4°C. Finally, liver mitochondria were resuspended in 250mM Sucrose and 10mM Tris/HCl at pH 7.4 and kept on ice. Skeletal muscles were carefully dissected, minced and homogenized in a glass Teflon potter in the homogenization buffer containing 140mM KCl, 20mM Hepes, 5mM MgCl₂, 2mM EGTA, 1mM ATP(Di-K) at pH 7.0 with 1% BSA and 2U/g of tissue of proteases (Sigma). After centrifugation at 500g/10min/4°C, the collected supernatant was further centrifuged at 9,000g/8min/4°C. Pellet was resuspended in the isolation medium (140mM KCl, 20mM Hepes, 5mM MgCl₂, 1mM EGTA at pH 7.0) and incubated on ice for 5min for myofibrillar re-polymerization. The two previous centrifuge steps were repeated and the final mitochondrial pellet resuspended in the isolation medium. Protein content was determined by the Lowry method (BioRad).

Live cell calcium imaging
RCaMP and ratiometric pericam were described in (Akerboom et al., 2013; Nagai et al., 2001). For imaging experiments, the cells were pre-incubated in a serum-free extracellular medium (ECM, 120 mM NaCl, 5 mM NaHCO₃, 10 mM Na-HEPES, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2 mM CaCl₂, 10 mM glucose, pH 7.4) containing 2% BSA. For SOCE experiments ER stores were depleted by 10 min pretreatment with 2 μM thapsigargin (Tg) in Ca²⁺-free ECM. For permeabilized cell experiments, the transfected cells were washed with a Ca²⁺-free ECM containing 100 μM EGTA/Tris and transferred to the imaging chamber in 1 ml intracellular medium (ICM, composed of 120 mM KCl, 10 mM NaCl, 1 mM KH₂PO₄, 20 mM Tris-HEPES at pH 7.2, 2 mM MgATP). Plasma membrane permeabilization was carried
out using saponin 40µg/ml. After 5 min permeabilization (35°C), the cells were washed into fresh ICM supplemented with 2 mM succinate, 10 µM EGTA/Tris. Fluorescence wide field imaging of [Ca^{2+}]_c and [Ca^{2+}]_m was carried out using a ProEM1024 EM-CCD (Princeton Instruments), fitted to Leica DMI 6000B inverted epifluorescence microscopes. When both [Ca^{2+}]_c and [Ca^{2+}]_m were recorded, with fura2 and mtipcam, 340/30nm, 380/20nm, and 490/20nm excitation filters were used with beam splitter 500 nm and emission filter 540/50nm and an image triplet was obtained in every 2s. [Ca^{2+}]_c and [Ca^{2+}]_m were also recorded respectively with RCaMP and mtrpcam alternating filter sets of ex:580/20 nm, bs595 nm, em: 630/60 nm and ex:415/20 nm, bs500nm and em:540/50 nm. The fura2 ratios were calibrated in terms of nM [Ca^{2+}]_c, whereas the ipcam, RCaMP or rpcam fluorescence at each time point was normalized to the initial fluorescence (F_0/F for the icam and F/F_0 for the others).

Fluorometric measurements of mitochondrial Ca^{2+} uptake, membrane potential and NADH
Isolated mitochondria (750µg) were resuspended in 1.5ml of ICM supplemented with proteases inhibitors (leupetin, antipain, pepstatin, 1mg/ml each), 2 mM MgATP, 2 µM Tg and maintained in a stirred thermostated cuvette at 35°C on a fluorimeter (DeltaRAM, PTI). Runs were performed in presence of 20 μM CGP-37157 and 1 mM Malate/Pyruvate. The extramitochondrial Ca^{2+} concentration [Ca^{2+}]_c was assessed using fura2 (1.5 µM) or furaFF (1 µM), whereas ΔΨ_m was measured with 1.5 µM TMRM. Fura2/furaFF and TMRM fluorescence were recorded simultaneously using 340-380 nm excitation and 500 nm emission, and 545 nm excitation and 580 nm emission, respectively. Complete dissipation of ΔΨ_m was elicited by addition of 2 µM FCCP. Calibration of the fura signal was carried out at the end of each measurement, adding 1 mM CaCl_2, followed by 10 mM EGTA/Tris, pH 8.5.

Protein extracts and Western Blotting
Protein lysates from cells or mitochondria were prepared in RIPA buffer supplemented with proteases inhibitors (leupetin, antipain, pepstatin, PMSF). Equivalent amounts of total protein (50µg) were separated electrophoretically by SDS-PAGE (10% TGX gels, BioRad) in reducing conditions and transferred to a PVDF membrane (Biorad). The latter was blocked in Odyssey blocking solution (Licor) for 1h at RT, and probed overnight at 4°C using the following primary antibodies: MICU1 (Sigma, HPA037480, 1:500), MICU2 (Abcam, ab101465, 1:500), MCU (Sigma, HPA016480, 1:500), MICU3 (Sigma, HPA024048, 1:1000), EMRE (Santa Cruz, sc-86337, 1:200) and Hsp70 (Thermo Scientific, MA3-028, 1:1000). Specificity of the antibodies was first confirmed through downregulation or overexpression experiments (data not shown). Membranes were scanned using an Odyssey scanner (Licor) after incubation with fluorescent secondary antibodies. Quantification was performed using ImageJ (NIH).

Co-immunoprecipitation
For co-IP experiments, HEKs cells were grown in T75 flasks. 36 hours post-transfection, cells were lysed in 1.5ml IP buffer (150mM NaCl, 20mM Hepes, 0.5mM EGTA, pH 7.4) supplemented with proteases inhibitors (leupetin, antipain, pepstatin, PMSF) and 0.5% Dodecyl-Maltoside. After centrifugation, lysates were split to be used for both FLAG and HA IP using respectively the Anti-DYKDDDDK-Agarose Beads (Clontech, #635686) and the Anti-HA–Agarose antibody beads (Sigma, A2095).

RNA isolation and qPCR
Total RNA were extracted from cells using TRIzol reagent (Ambion) and then treated with RQ1 DNase (Promega). Mice liver and heart RNA were isolated using the animal tissue RNA purification kit (Norgen) according to the manufacturer’s instructions. Quantification was done on the nanophotometer Pearl (Implen). Then cDNA was synthesized using SuperScript III (Invitrogen) and used for qPCR reactions using SYBR Green (Invitrogen) on an ABI Prism 7000 sequence detection system (Life Technologies). Data were analyzed using the comparative 2-ΔΔCt method. Ct of the gene of interest was normalized to that of β-actin for heart and liver comparison. Primers used are listed below:
| Target | Forward | Reverse |
|--------|---------|---------|
| Human MICU1 | ACAGTGCTAAAGTGGAGC | GTTTGGGTAAAGCGAAGTCC |
| Mice MICU1 | AACAGCAAGAAGCTGACAC | CTCATTGGGCTTATGGAG |
| Mice MICU2 | GGAGCGTAAAACACTGGTC | GTAAGCAAGAAAGATACTCGG |
| Mice MCU | TACCTCACCAGATGCCGGTC | GTCTCCTAACCCTCTCCAC |
| Mice MCUb | AGTTACCTTCTTCTGTCGTTTGCAG | CAGGGATTCTGTAGCCTGAGCAAGG |
| β-actin | CAACACCCCAGCCATG | GTCACGCACGATTCCC |

**Echocardiography**
To measure global cardiac function, echocardiography was performed with the VisualSonics VeVo 2100 imaging system in anesthetized animals (1.5% [vol/vol] isoflurane).

**SUPPLEMENTAL BIBLIOGRAPHY**
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