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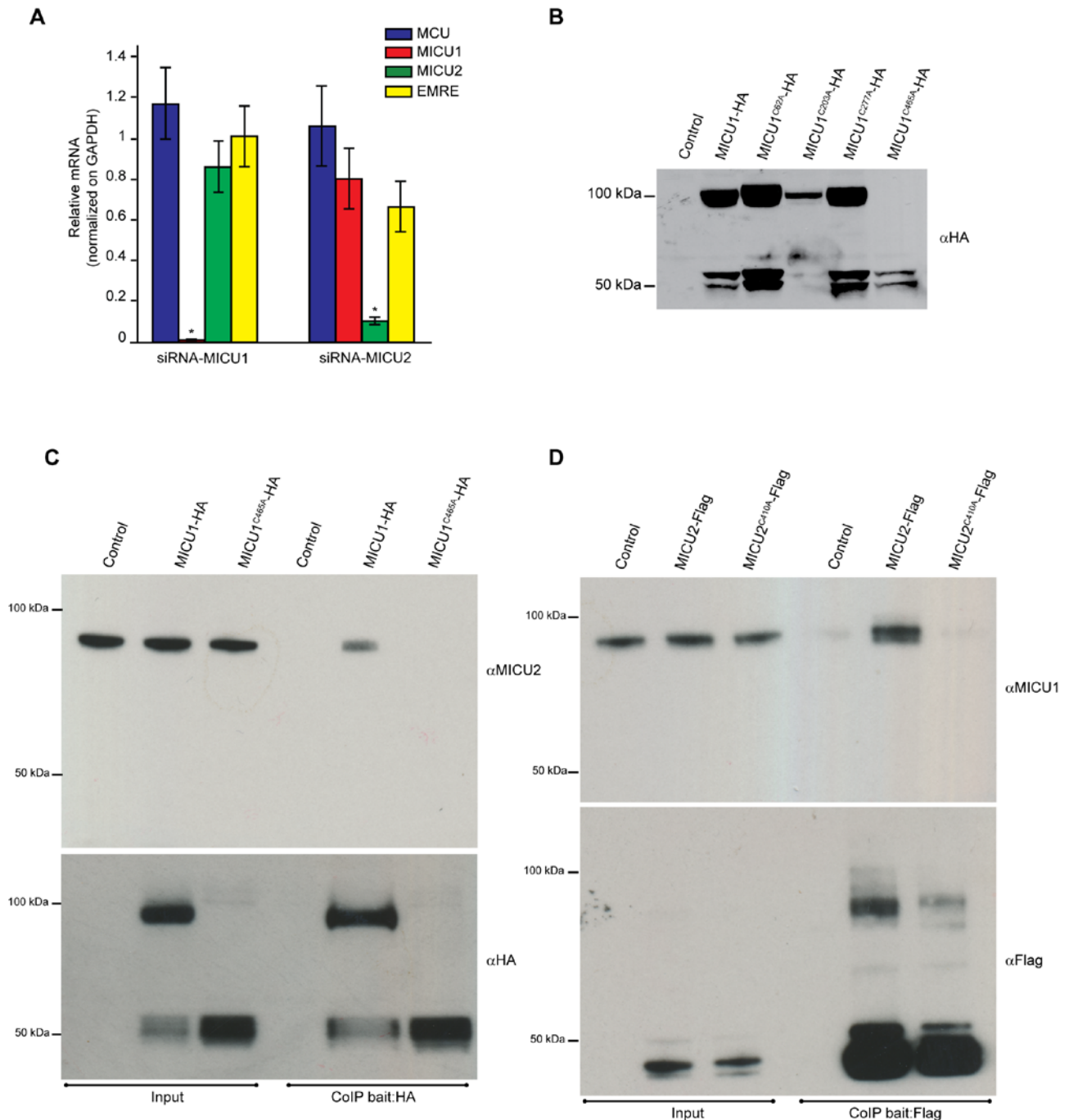
**Supplemental Information**

**MICU1 and MICU2 Finely Tune the Mitochondrial Ca<sup>2+</sup> Uniporter by Exerting Opposite Effects on MCU Activity**

Maria Patron, Vanessa Checchetto, Anna Raffaello, Enrico Teardo, Denis Vecellio Reane, Maura Mantoan, Veronica Granatiero, Ildikò Szabò, Diego De Stefani, and Rosario Rizzuto

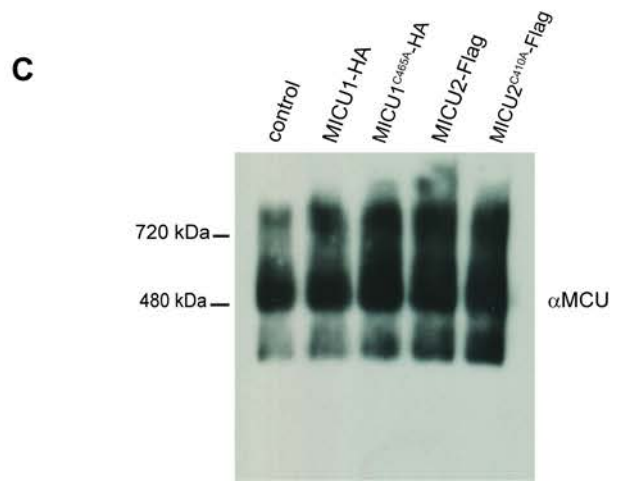
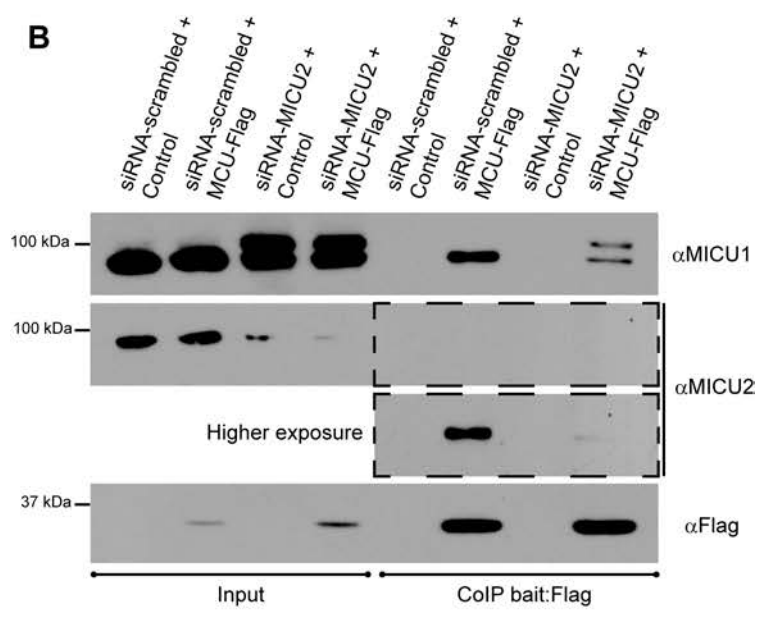
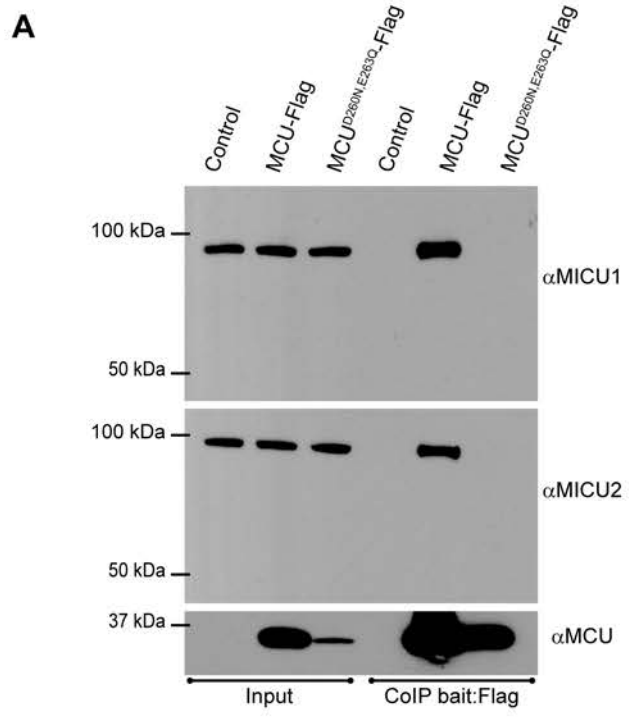
## Supplemental information

### Supplemental figures:

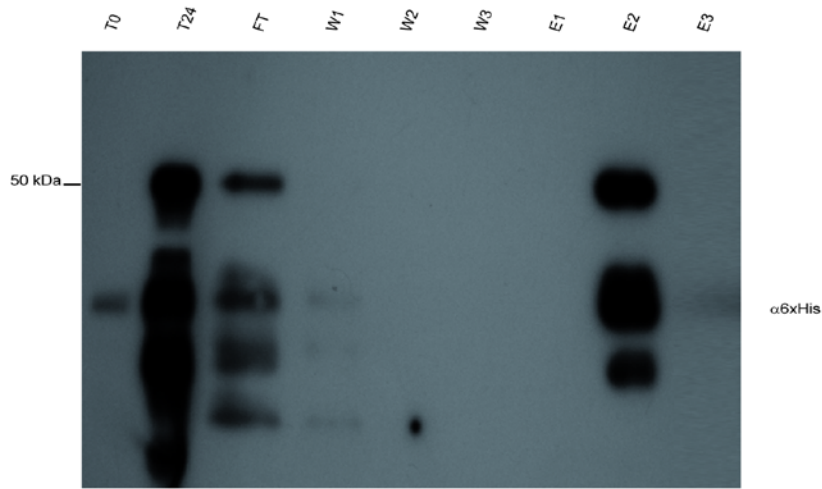
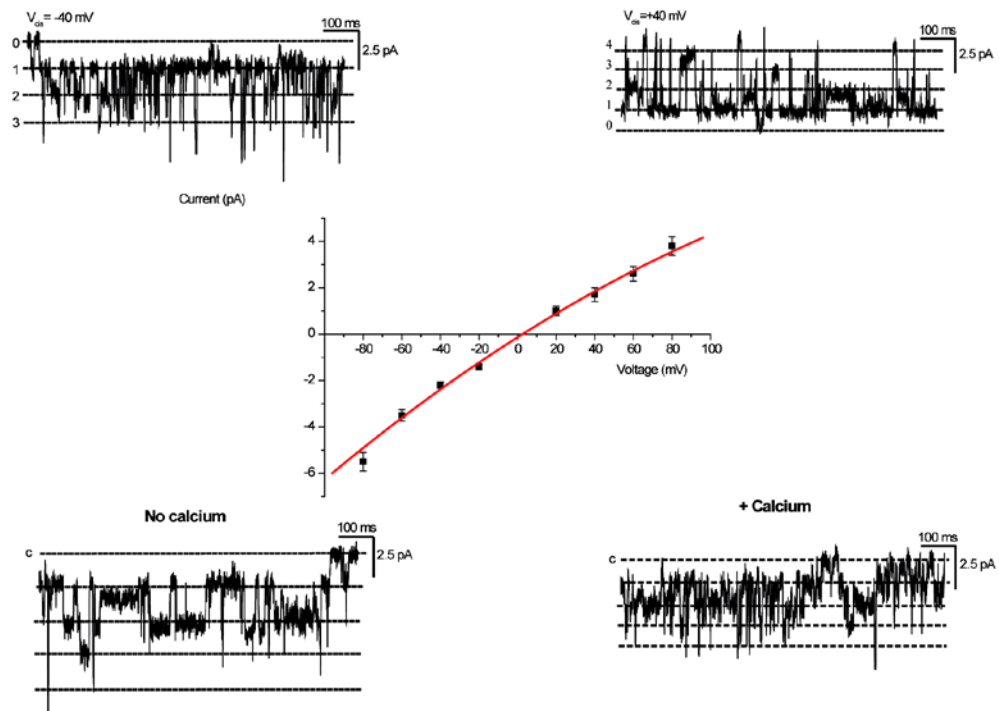
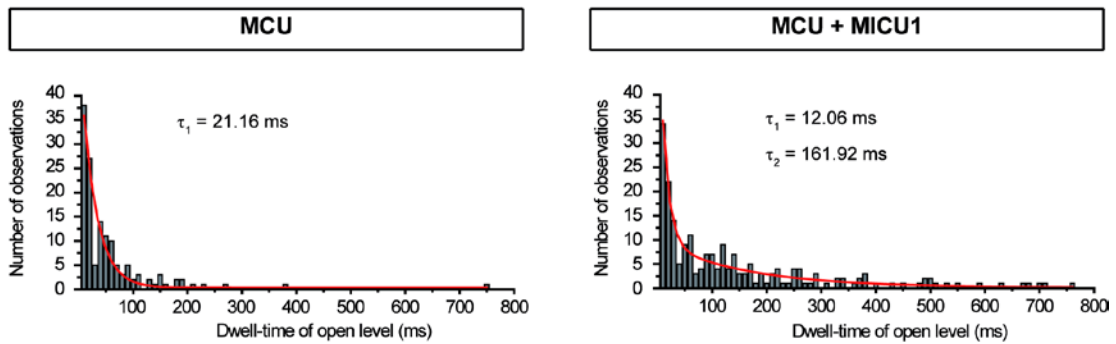
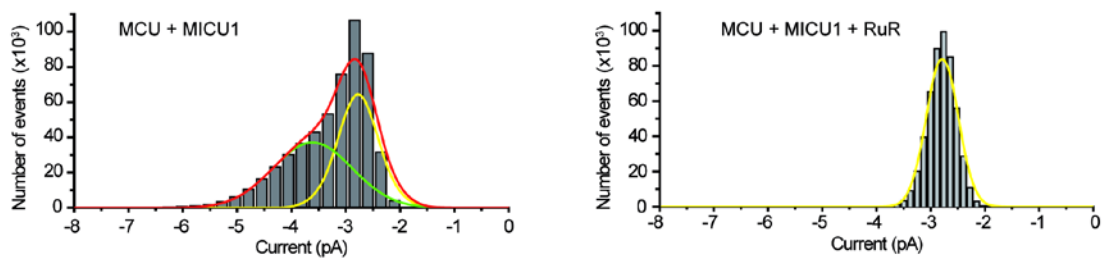


**Figure S1. Biochemical characterization of MICU1/MICU2 interaction, related to Figure 2.** (A) HeLa cells were transfected with siRNA-MICU1 or siRNA-MICU2. mRNA was extracted and quantitative Real Time PCR for MICU1, MICU2, MCU and EMRE was performed as detailed in Experimental procedures section. Data are presented as the normalized ratio (compared to siRNA-scrambled)  $\pm$  S.D. (for siRNA-MICU1:  $1.170 \pm 0.174$ , MCU;  $0.011 \pm 0.001$ , MICU1;  $0.860 \pm 0.128$ , MICU2;  $1.011 \pm 0.150$ , EMRE; for siRNA-MICU2:  $1.060 \pm 0.194$ , MCU;  $0.801 \pm 0.147$ , MICU1;  $0.104 \pm 0.019$ , MICU2;  $0.663 \pm 0.122$ ,

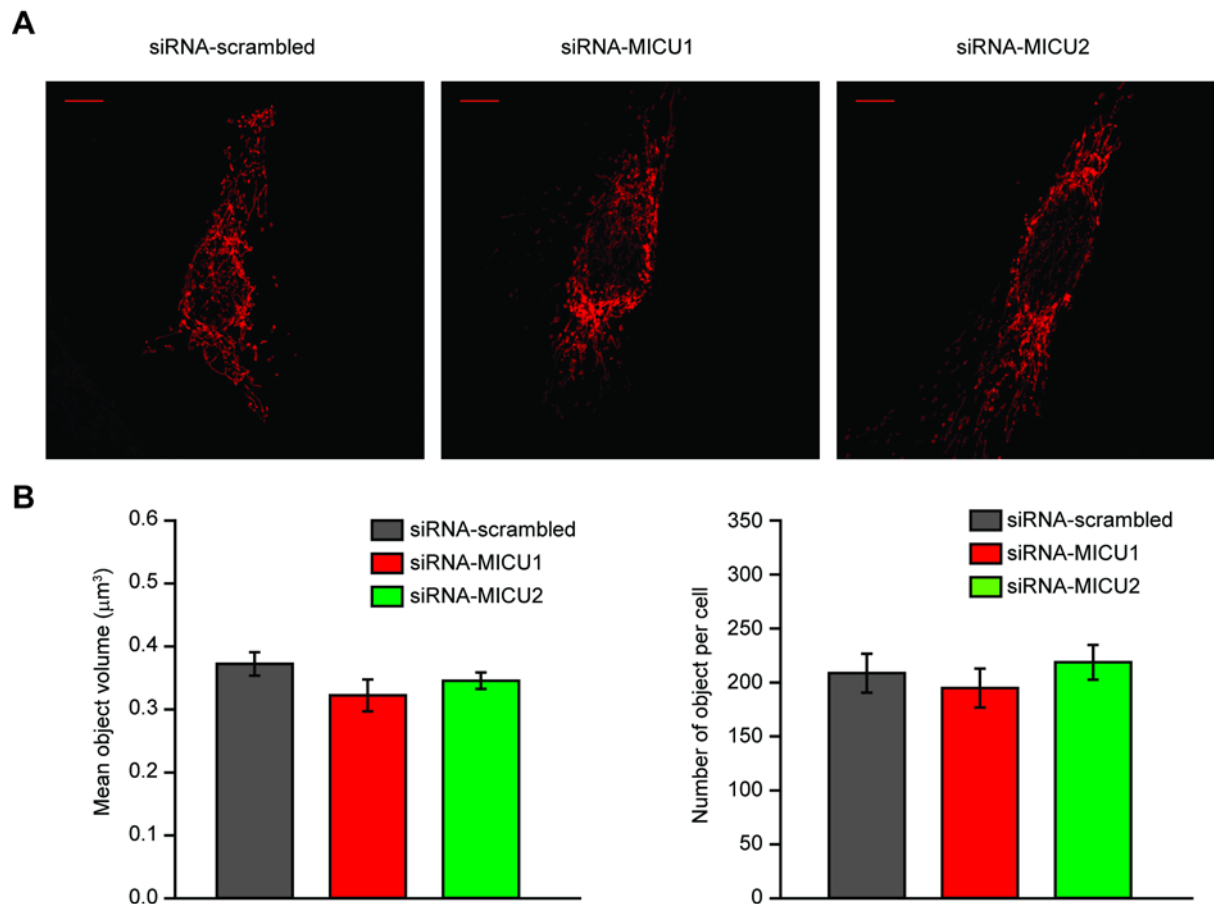
EMRE).\* indicates  $p < 0.05$  compared to control. (B, C and D) HeLa cells were transfected with the indicated constructs. (B) HeLa cells were harvested after 48 hours of transfection, total protein were extracted and subjected to non-reduced SDS-PAGE and Western blotting analysis with antibodies  $\alpha$ HA. (C) HA-tagged MICU1 constructs were immunoprecipitated from whole cell lysate with a specific  $\alpha$ HA-Agarose-conjugated antibody. The precipitated proteins were immunoblotted with  $\alpha$ MICU2 and  $\alpha$ HA antibodies. (D) Flag-tagged MICU2 constructs were immunoprecipitated from whole cell lysate with a specific  $\alpha$ Flag-Agarose-conjugated antibody. The precipitated proteins were immunoblotted with  $\alpha$ MICU1 and  $\alpha$ Flag antibodies. SDS-PAGE was performed in the presence of DTT.



**Figure S2. Structural requirements for MICU1/MICU2 interaction with MCU, related to Figure 2.** (A) HeLa cells were transfected with MCU-Flag and MCU<sup>D260N,E263Q</sup>-Flag. Whole cell lysate was immunoprecipitated using specific monoclonal  $\alpha$ Flag-agarose-conjugated antibody. The precipitated proteins were immunoblotted with  $\alpha$ MICU1,  $\alpha$ MICU2 and  $\alpha$ Flag antibodies. SDS-PAGE was performed in the absence of DTT. (B) HeLa cells were transfected with the indicated constructs. Flag-tagged MCU was immunoprecipitated from whole cell lysate with a specific  $\alpha$ Flag-Agarose-conjugated antibody. The precipitated proteins were immunoblotted with  $\alpha$ MICU1,  $\alpha$ MICU2 and  $\alpha$ FLAG antibodies. Please note that MCU can interact with both MICU1/MICU2 heterodimer (lower band in last lane) and MICU1/MICU1 homodimer (higher band in last lane). SDS-PAGE was performed in the absence of DTT. (C) Crude mitochondrial fractions were isolated from HeLa cells were transfected with the indicated constructs. Protein complexes were then extracted and blue native PAGE was performed as indicated in the Experimental procedures section. Proteins were then transferred on PVDF membrane and immunoblotted with  $\alpha$ MCU antibody.

**A****B****C****D**

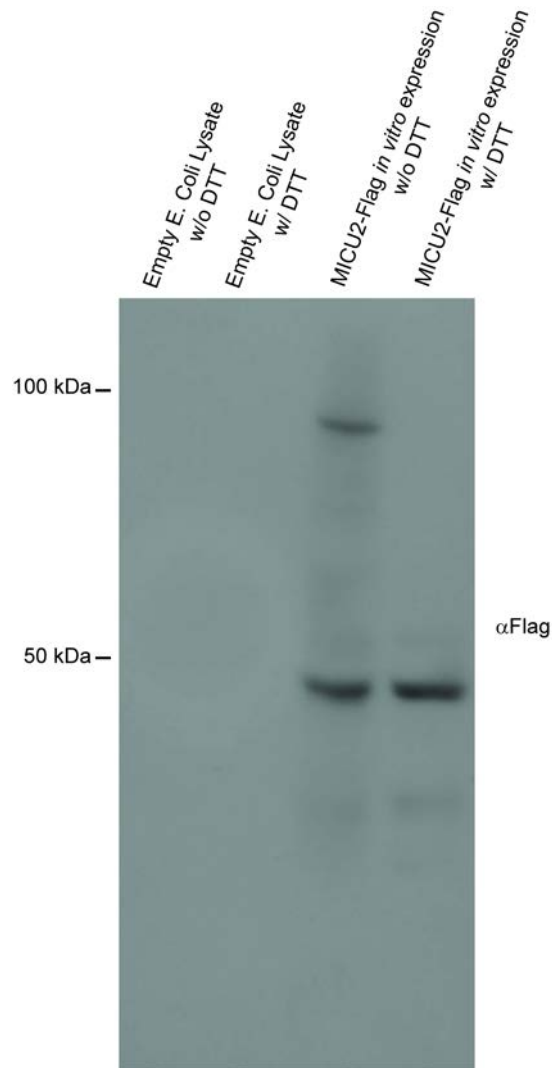
**Figure S3. MICU1 activates MCU in planar lipid bilayer, related to Figure 3.** (A) Induction and purification of MICU1 in *E. coli*. Bacteria were harvested before (T0) and after induction (T24) to check for the expression of the protein. After sonication, total bacteria lysate was passed through the Nickel-affinity column (Flow-Through); after washing (W1-W3), protein was eluted with an imidazole gradient (E1-E3). All samples were blotted and developed with antibody  $\alpha$ 6xHis. 30  $\mu$ l of eluted fractions/lane were loaded. (B) MCU allows flow of sodium in the absence of calcium (low divalent solution). Representative current traces, recorded at the indicated voltages with *in vitro* expressed MCU are shown. Current-voltage relationship reveals a  $55 \pm 5$  pS (n=3) conductance at negative voltages. Mean current values  $\pm$  S.D. are reported in function of the applied voltage. Values were determined either by measuring current amplitudes of single channel openings by using the PCLAMP8.0 program set (n  $\geq$  50 for each voltage of 2 independent experiments). In lower panel current trace recorded at -50 mV in sodium is shown from another experiment before (left) and after (right) addition of 16 nM  $\text{Ca}^{2+}$  to the *cis* compartment. Free calcium concentration was calculated with the WebMaxC v2.2 program. Please note reduction of the sodium current flowing through the channels in the presence of calcium is a known characteristic of calcium channels and is compatible with the behavior of MCU in whole-mitoplast configuration. In a previous report using patch clamp on mitoplasts (Fieni et al., 2012) the authors reported a block of sodium current across MCU by 160 nM calcium. We observe a very small sodium current in the presence of 1  $\mu$ M calcium (Figure 1). However, please note that we use an *in vitro* expressed MCU in an *in vitro* bilayer system and factors determining the affinity of calcium binding might be different in the two systems (e.g. for voltage-gated calcium channels it is well known that ion permeation properties can be altered by protonation of the P-loop glutamates and due to modulation by G-protein (Varadi et al., 1999)). (C) Open time histogram after addition of MICU1 was best fitted by the sum of two exponential components, giving open time constants of 12.06 and 161.9 ms. (B) Amplitude histograms show baseline current (yellow) and MCU current (green) in the presence of MICU1 (left histogram), while histogram on the right was obtained following addition of Ruthenium Red (1  $\mu$ M). Consecutive traces of 60 s were analyzed. Although at 1  $\mu$ M concentration RuR can inhibit various cationic channels, please note that in the expression system and bilayer used by us MCU is the only channel present.



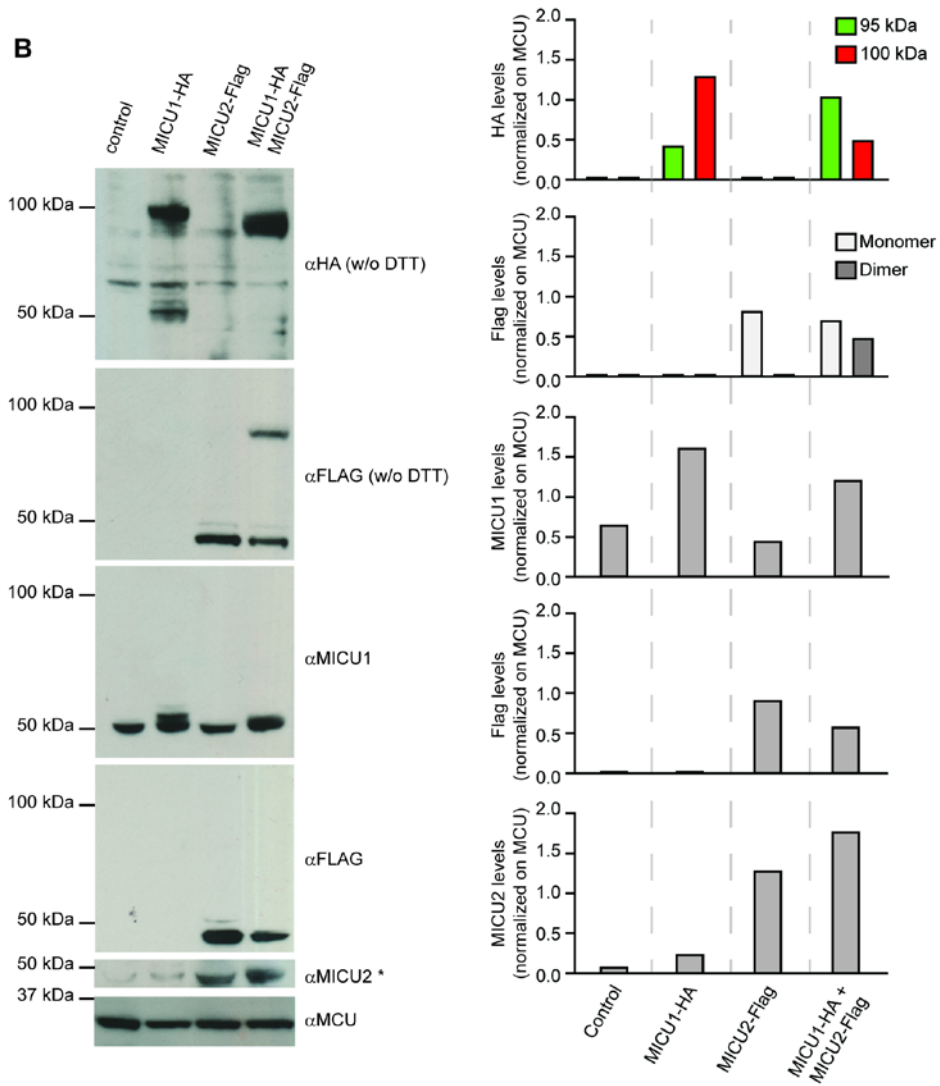
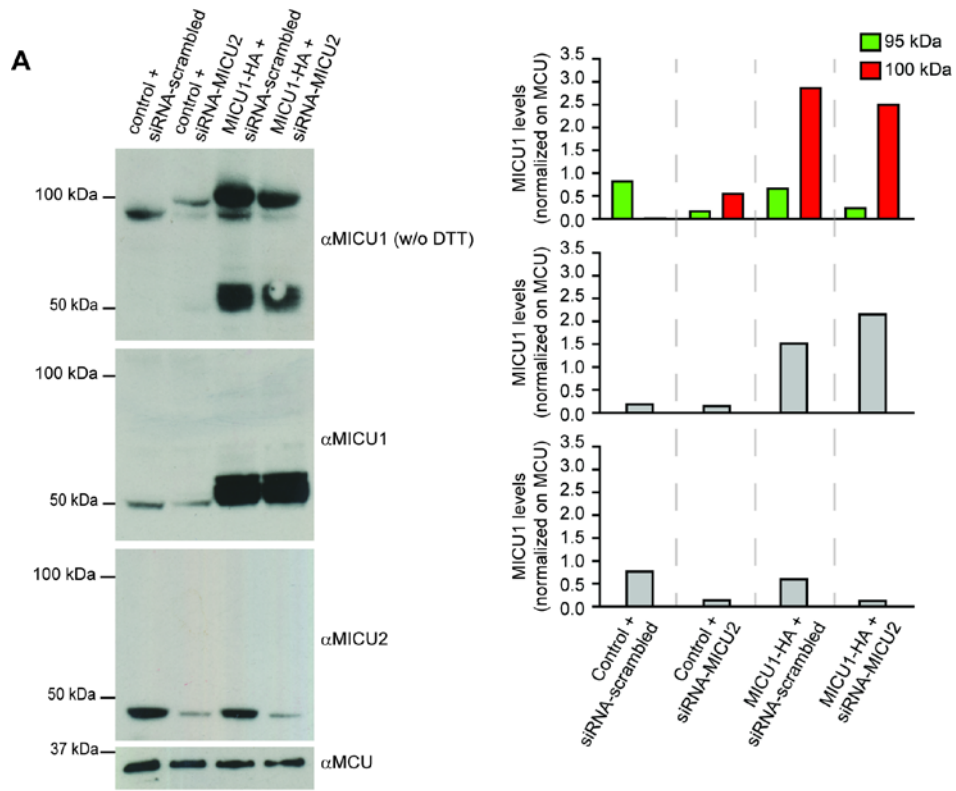
**Figure S4. MICU1 and MICU2 silencing does not alter mitochondrial morphology, related to Figure 5.**

(A) Representative images of HeLa cells expressing a mitochondrial targeted DsRed transfected with the indicated siRNA. Scale bars indicate 10  $\mu\text{m}$ . (B) Bar graphs showing the mean volume of individual mitochondria ( $0.372 \pm 0.065 \mu\text{m}^3$ , siRNA-scrambled;  $0.322 \pm 0.088 \mu\text{m}^3$ , siRNA-MICU1;  $0.346 \pm 0.046 \mu\text{m}^3$ , siRNA-MICU2) and the number of mitochondria per cell ( $209 \pm 18$ , siRNA-scrambled;  $195 \pm 18$ , siRNA-MICU1;  $219 \pm 16$ , siRNA-MICU2).





**Figure S5. Expression and purification of MICU2, related to Figure 5.** MICU2-Flag was expressed *in vitro* using the RTS 100 *E.coli* Disulfide Kit (Roche). Proteins were then processed for SDS-PAGE and Western blot and probed with  $\alpha$ Flag antibody. Where indicated, proteins were treated with 30 mM DTT.



**Figure S6. MICU1 and MICU2 expression levels, related to Figure 6.** HeLa cells were transfected with the indicated constructs. (A) Western blot (left panels) and relative densitometric analysis (right panels) showing MICU1, MICU2 and MCU expression levels when MICU1 is overexpressed in a MICU2 silenced background. Where indicated, DTT was omitted in order to visualize MICU dimerization. (B) Western blot (left panels) and relative densitometric analysis (right panels) showing MICU1, MICU2 and MCU expression levels when MICU1 and MICU2 are overexpressed alone or in combination. Where indicated, DTT was omitted in order to visualize MICU dimerization. Please note that, in order to test MICU2 expression levels after overexpression, we used an antibody (marked with an asterisk) that can recognize both the endogenous (human) as well as the overexpressed (mouse) MICU2 isoforms.

## Supplemental table

Supplemental table 1

Experiment	Measurement	Mean $\pm$ S.D.	n	Figure
<b>GFP + mCherry</b>	FRET efficiency (%)	0.736 $\pm$ 0.980	18	1B
<b>MCU-GFP + MCU-mCherry</b>	FRET efficiency (%)	17.871 $\pm$ 8.260	14	1B
<b>MICU1-GFP + MICU2-mCherry</b>	FRET efficiency (%)	5.572 $\pm$ 4.704	29	1B
<b>MICU2-GFP + MICU2-mCherry</b>	FRET efficiency (%)	6.700 $\pm$ 4.105	9	1B
<b>MICU2-GFP + MICU1-mCherry</b>	FRET efficiency (%)	11.153 $\pm$ 3.998	15	1B
<b>MICU1-GFP + MICU2-mCherry</b>	FRET efficiency (%)	5.560 $\pm$ 3.942	10	1B
<b>Control</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	98.777 $\pm$ 10.17	16	4A
<b>MICU1</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	189.825 $\pm$ 29.61	16	4A
<b>siRNA-scrambled</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	60.823 $\pm$ 12.64	16	4B
<b>siRNA-MICU1</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	93.974 $\pm$ 11.71	16	4B
<b>siRNA-scrambled</b>	474/410nm ratio	1.956 $\pm$ 0.931	30	4C
<b>siRNA-MICU1</b>	474/410nm ratio	8.246 $\pm$ 1.446	30	4C
<b>siRNA-scrambled</b>	Ca <sup>2+</sup> uptake speed ( $\mu$ mol/sec)	0.029 $\pm$ 0.014	8	4D
<b>siRNA-MICU1</b>	Ca <sup>2+</sup> uptake speed ( $\mu$ mol/sec)	0.261 $\pm$ 0.133	8	4D
<b>siRNA-scrambled</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	69.249 $\pm$ 7.140	16	5A
<b>siRNA-MICU2</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	101.508 $\pm$ 12.236	16	5A
<b>Control</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	87.083 $\pm$ 6.25	16	5B
<b>MICU2</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	72.523 $\pm$ 9.479	16	5B
<b>Control + siRNA-scrambled</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	70.597 $\pm$ 5.729	18	6A
<b>Control + siRNA-MICU2</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	124.170 $\pm$ 8.450	18	6A
<b>MICU1 + siRNA-scrambled</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	100.044 $\pm$ 8.196	18	6A
<b>MICU1 + siRNA-MICU2</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	179.636 $\pm$ 17.847	18	6A
<b>Control</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	102.878 $\pm$ 20.969	16	6B
<b>MICU1</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	159.291 $\pm$ 17.427	16	6B
<b>MICU2</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	79.809 $\pm$ 19.934	16	6B
<b>MICU1 + MICU2</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	113.524 $\pm$ 21.337	16	6B
<b>Control</b>	474/410nm ratio	1.950 $\pm$ 0.778	30	6C
<b>MCU</b>	474/410nm ratio	7.328 $\pm$ 1.211	30	6C
<b>MCU + MICU1</b>	474/410nm ratio	5.813 $\pm$ 1.125	30	6C
<b>MCU + MICU1 + MICU2</b>	474/410nm ratio	1.328 $\pm$ 0.060	30	6C
<b>Control</b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	98.777 $\pm$ 10.17	8	6D
<b>MICU1<sup>EFmut</sup></b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	69.198 $\pm$ 10.141	8	6D
<b>MICU2<sup>EFmut</sup></b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	36.281 $\pm$ 15.560	8	6D
<b>MICU1<sup>EFmut</sup> + MICU2<sup>EFmut</sup></b>	[Ca <sup>2+</sup> ] <sub>mt</sub> peak value ( $\mu$ M)	20.165 $\pm$ 13.715	8	6D

Table S1. Descriptive statistics for the experiments shown in Figures 1, 4, 5 and 6.

## Supplemental experimental procedures

### *Chemicals, cell culture and transfection*

All chemicals were purchased from Sigma-Aldrich, unless specified.  $\alpha$ Flag (IF, 1:100),  $\alpha$ HA (IF, 1:100),  $\alpha$ MICU1 (WB, 1:1000),  $\alpha$ MICU2 (WB, 1:1000) and  $\alpha$ MCU (WB, 1:1000) were purchased from Sigma-Aldrich.  $\alpha$ Flag (WB, 1:1000),  $\alpha$ HA (WB, 1:1000) was purchased from Cell Signaling.  $\alpha$ TOM20 (IF, 1:100) was purchased from Santa Cruz. A  $\alpha$ MICU2 antibody from Abcam (1:250) recognizing both the human and mouse MICU2 isoforms was used in figure S6. Secondary, HRP-conjugated antibodies (WB, 1:5000) were purchased from BioRad. Secondary, AlexaFluor-conjugated antibodies (IF, 1:500) were purchased from Life Technologies. Please note that MICU2 antibody is specific for the human isoform and thus not recognized the MICU2-Flag mouse construct.

In all the experiments HeLa cells were used (ATCC Number: CCL-2). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies), supplemented with 10% fetal bovine serum (FBS) (Life Technologies) and transfected with a standard calcium-phosphate procedure. For aequorin measurements, the cells were seeded 24 hours before transfection onto 13 mm glass coverslips and allowed to grow to 50% confluence before transfection. For imaging experiments, cells were seeded 24 hours before transfection onto 24 mm glass coverslips and allowed to grow to 50% confluence before transfection, unless otherwise specified.

### *Constructs and siRNAs*

Mouse MICU1 (NM\_144822) was amplified from mouse skeletal muscle cDNA by PCR using the following primers:

For the cloning of MICU1-HA in pcDNA3.1:

fw: 5'-CGGATCCGCCACCATGTTTCGTCTTAACACCCT-3'

rv:

3'-

GCTCGAGTCACAGGGAAGCGTAGTCAGGCACATCGTAGGGGTATTTGGGCAGAGCAAAGTCCC  
-5'

The PCR fragment was cloned into BamHI and XhoI sites in pcDNA3.1 (Life Technologies).

For the cloning of MICU1 in pEGFP-N1 and pmCherry-N1:

fw: 5'-CCTCGAGATGTTTCGTCTTAACACCCT-3'

rv: 5'-CGGATCCCGTTTGGGCAGAGCAAAGTCCC-3'

The PCR fragment was cloned into XhoI and BamHI sites in pEGFP-N1 and pmCherry-N1 (Clontech).

For the cloning of MICU1 in pET-28A(+):

fw: 5'-AGGATCCATGTTTCGTCTTAACACCCT-3'

rv: 5'-ACTCGAGCTATTTGGGCAGAGCAAAGT-3'

The PCR fragment was cloned into BamHI and XhoI sites in pET-28A(+) (Novagen).

Mouse MICU2-Flag (NM\_028643.3) and 2mtGCaMP6m were obtained by custom gene synthesis (Life Technologies).

For cloning in pIVEX2.3d (Roche), MICU2-Flag was subcloned by using NcoI and XhoI.

The generation of the pcDNA3.1-MICU1<sup>C62A</sup>-HA, pcDNA3.1-MICU1<sup>C203A</sup>-HA, pcDNA3.1-MICU1<sup>C277A</sup>-HA, pcDNA3.1-MICU1<sup>C465A</sup>-HA, pcDNA3.1-MICU1<sup>D233A,E244K,D423A,E434K</sup>-HA (MICU1<sup>EFmut</sup>), pcDNA3.1-MICU2<sup>D372A</sup>-Flag (MICU2<sup>EFmut</sup>) and pcDNA3.1-MICU2<sup>C410A</sup>-Flag mutant was performed by mutagenesis PCR using the wild type pcDNA3.1-MICU1-HA and pcDNA3.1-MICU2-Flag vectors as template and the mutagenesis primers:

For pcDNA3.1-MICU1<sup>C62A</sup>-HA:

5'-CTCACGCAGAATCTCCACCAGCCGTTAACAGCAAGAAGCCTGAC-3'

For pcDNA3.1-MICU1<sup>C203A</sup>-HA:

5'-TCTTCTACAGCCTTGGAGAGGCTGGGCTCATCTCCTTCTCTG-3'

For pcDNA3.1-MICU1<sup>C277A</sup>-HA:

5'-ACACCCTCAAGTCTGGCTTAGCTTCGGCCCTCACGACCTAC-3'

For pcDNA3.1-MICU1<sup>C465A</sup>-HA:

5'-TCATGCAGGCCATGTGGAAAGCTGCCCAAGAAACTGCCTGG-3'

For pcDNA3.1-MICU1<sup>D233A,E244K,D423A,E434K</sup>-HA:

5'-

TGAAATTGCTTTCAAGATGTTTGCCTTGAATGGAGACGGAGAGGTAGACATGGAGAAGTTTGA  
GCAGGTTTCAGAGC-3'

5'-

GTGGTGTTCGCGCTCTTTGCCTGTGATGGCAATGGGGAGCTGAGCAACAAGAAGTTCGTTTCCA  
TCATGAAGC-3'

For pcDNA3.1-MICU2<sup>D372A</sup>-Flag:

5'-GACACCGTCTTCAAGATCTTTGCCCTGGACGGGGACGAGTGC-3'

For pcDNA3.1-MICU2<sup>C410A</sup>-Flag:

5'-AGTGTGCAAGAATACTGGAAGGCTGTGAAGAAGGAAAGCATCAAG-3'

To silence MICU1 specific leading siRNA sequences were designed:

siRNA-MICU1#1: 5'-UCUGAAGGGAAAGCUGACAAU-3'

siRNA-MICU1#2: 5'-GACUUCGCUUUACCCAAACAG-3'

siRNA-MICU1#3: 5'-UGAACAGGUUCAGAGCAUCAU-3'

siRNA-MICU1#4: 5'-UCAAGAAGCACUUCAAAGAAG-3'

All the above sequences were able to significantly downregulate MICU1 expression levels and increase mitochondrial calcium uptake. For all the experiments shown in the text, siRNA-MICU1#1 was used.

To silence MICU2 specific leading siRNA sequences were designed:

siRNA-MICU2#1: 5'-CACGAGACUUCUCUCUCAG-3'

siRNA-MICU2#2: 5'-AGUAAAAGAAGUCUGGAAACA-3'

siRNA-MICU2#3: 5'-AUGAAACUGGAUAUCAGGAAG-3'

siRNA-MICU2#4: 5'-AGAAGUCUGGAAACAAGCUGG-3'

All the above sequences were able to significantly downregulate MICU2 expression levels and increase mitochondrial calcium uptake. For all the experiments shown in the text, siRNA-MICU2#1 was used.

All the siRNAs and the non-targeting siRNA (siRNA-scrambled, MISSION® siRNA Universal Negative Control #1, cat no. SIC001) were purchased from Sigma-Aldrich.

MCU-Flag, MCU-GFP, MCU-mCherry and MCU<sup>D260N, E263Q</sup>-Flag were previously described (Raffaello et al., 2013).

### ***RNA extraction, reverse transcription and Quantitative Real Time PCR***

For the expression analysis of MCU, MICU1, MICU2 and EMRE HeLa cells were used. At least 3 samples were prepared for each condition. Total RNA was extracted from 15–60 mg of  $1 \times 10^6$  HeLa cells using the SV Total RNA Isolation Kit (Promega) following manufacturer instructions. The RNA was quantified with an Eppendorf Bio photometer Plus. From an equal amount of total RNA of each sample, complementary DNA was generated with a cDNA synthesis kit (SuperScript II, Invitrogen) and analyzed by real-time PCR using the SYBR green chemistry (Bio-Rad). The primers were designed and analyzed with Primer3 (Rozen and Skaletsky, 2000). Identity of the amplicons was confirmed by their dissociation profiles and gel analysis. Real-time PCR standard curves were constructed by using serial dilutions of cDNAs of the analyzed samples, using at least four dilution points and the efficiency of all primer sets was between 95 and 105%. The housekeeping genes Beta Actin and GAPDH were used as an internal control for cDNA quantification and normalization of the amplified products. Real-time PCR primer sequences were as follows:

To amplify MCU, MICU1, MICU2 and EMRE in HeLa cells:

GAPDH-fw: 5' -CACCATCTTCCAGGAGCGAG-3'

GAPDH-rv: 5' -CCTTCTCCATGGTGGTGAAGAC-3'

These primers amplify a fragment of 133 base pairs.

Beta Actin-fw: 5' -ATAGCACAGCCTGGATAGCAACGTAC-3'

Beta Actin-rv: 5' -CACCTTCTACAATGAGCTGCGTGTG-3'

These primers amplify a fragment of 133 base pairs.

MCU-fw: 5' -GCAGAATTTGGGAGCTGTTT-3'

MCU-rv: 5' -GTCAATTCCCCGATCCTCTT-3'

These primers amplify a fragment of 195 base pairs.

MICU1-fw: 5' -GAGGCAGCTCAAGAAGCACT-3'

MICU1-rv: 5' -CAAACACCACATCACACACG-3'

These primers amplify a fragment of 195 base pairs.

MICU2-fw: 5' -GGCAGTTTTACAGTCTCCGC-3'

MICU2-rv: 5' -AAGAGGAAGTCTCGTGGTGTC-3'

These primers amplify a fragment of 195 base pairs.

EMRE-fw: 5' -TGTCGGGACACTCATTAGCA-3'

EMRE-rv: 5' -GCTGATAGGGAAGGCAGAGA-3'

These primers amplify a fragment of 155 base pairs.

### ***Co-immunoprecipitation***

For co-immunoprecipitation experiments, HeLa cells were grown in 10 cm Petri dishes and transfected with the calcium-phosphate procedure with pcDNA3.1, pcDNA3.1-MCU-Flag, pcDNA3.1-MCU<sup>D260N,E263Q</sup>-Flag, pcDNA3.1-MICU2-Flag, pcDNA3.1-MICU2<sup>C410A</sup>-Flag, pcDNA3.1-MICU1<sup>C465A</sup>-HA as indicated. After 36 hours of expression cells were lysate in an appropriate volume of lysis buffer (125 mM NaCl, 25 mM TRIS-Cl pH7.4, 1 mM EGTA-Tris pH 7.4, 0.5% n-Dodecyl  $\beta$ -D-maltoside, Complete EDTA-free protease inhibitor mixture and PhosSTOP Phosphatase Inhibitor Cocktail (Roche Applied Science)). Whole cell lysate was precleared for 30' with a control agarose resin (Pierce). 1 mg of pre-cleared proteins from the indicated conditions was incubated with monoclonal  $\alpha$ Flag or  $\alpha$ HA agarose-conjugated antibody (Sigma) and the co-immunoprecipitation was performed following manufacturer instructions. After three 10' washes in lysis buffer, the bait was eluted in a non-reducing Laemli sample buffer and denatured for 5' at 90°C. The precleared lysate (Input) and the immunoprecipitated (CoIP) samples were separated by SDS-PAGE, transferred to Hybond-C Extra membrane (Amersham) and developed according to standard procedures (see below). The same membrane in each panel was stripped for 10' in StripABlot stripping buffer (Euroclone) and probed with different antibodies as indicated.

### ***SDS-PAGE and Western Blot***

For protein extraction, HeLa cells were grown on 10 cm petri dishes and transfected with the indicated constructs as described above. After 24 or 48 hours, cells were washed twice with PBS, scraped, harvested and resuspended in the appropriate volume of RIPA buffer (125 mM NaCl, 25 mM TRIS-Cl pH 7.4, 1 mM EGTA-TRIS pH 7.4, 1% Triton-X100, 0.5% sodium deoxycholate, 0.1% SDS and Complete EDTA-free protease inhibitor mixture (Roche Applied Science)) and briefly sonicated. Crude extracts were centrifuged at 15000xg for 10' to remove debris, and proteins in the supernatant were quantified using the BCA Protein Assay Kit (Pierce). 40  $\mu$ g of proteins were dissolved in LDS sample buffer (Life Technologies), heated for 5' at 70°C and loaded on 4-12% Bis-Tris NuPage gels (Life Technologies). Where indicated, proteins were reduced with 100mM DTT and denatured for 5' at 90°C, unless specified. After electrophoretic separation, proteins were transferred onto nitrocellulose membranes and probed with the indicated antibodies. Isotype matched, horseradish peroxidase-conjugated secondary antibodies (BioRad) were used followed by detection by chemiluminescence (SuperSignal Pico, Pierce). Densitometry was performed using the built-in function present in Fiji image processing package based on ImageJ (Schindelin et al., 2012). Western blots shown in figures are representative of at least 5 different independent experiments.



### ***Blue Native PAGE***

For protein extraction, HeLa cells were grown on 15 cm petri dishes and transfected with the indicated constructs as described above. After 36 hours, cells were washed twice with PBS, scraped, harvested and mitochondria were isolated through differential centrifugation as previously described (Frezza et al., 2007). The mitochondrial fractions were lysed in the appropriate volume of lysis buffer (50 mM NaCl, 50 mM BisTris, 6 N HCl, 10% w/v glycerol, 0.001% Ponceaus S, 0.5% n-Dodecyl  $\beta$ -D-maltoside, pH7.2). Crude extracts were centrifuged at 15000xg for 10' to remove debris, and proteins in the supernatant were quantified using the BCA Protein Assay Kit (Pierce). 20  $\mu$ g of proteins were dissolved in 1x NativePAGE sample buffer (Life Technologies) and loaded on a 4-16% Novex NativePAGE Bis-Tris Gel System (Life Technologies). After electrophoretic separation, proteins were transferred onto PVDF membranes and probed with the indicated antibodies. Isotype matched, horseradish peroxidase-conjugated secondary antibodies (BioRad) were used followed by detection by chemiluminescence (SuperSignal Pico, Pierce).

### ***Protein expression and purification***

*E.coli*: DE3 competent cells (Stratagene) were transformed with pET28A(+)-MICU1. Induction was performed (O.D.600 0.4) with 0.35 mM IPTG for 24 hours.

Purification of MICU1: after sonication in Buffer A (50 mM Na-phosphate, pH 7.4, 300 mM NaCl + protease inhibitor cocktail), *E. coli* total lysate was solubilized with Decy- $\beta$ -D-maltopyranoside (2.5%) for 3 hours at room temperature. After centrifugation, the supernatant was loaded on a Nickel chromatography column (HIS-Select Nickel affinity gel, Sigma). Column was washed with buffer A and the protein was eluted with imidazole gradient (50-200 mM). Fractions of 500  $\mu$ l were collected and dialyzed against buffer A. A concentration of 0.025% of detergent was maintained throughout the purification.

*In vitro*: *in vitro* expression of pIVEX1.4WG:MCU was performed by using Wheat Germ CECF Kit (Roche). After expression, the MCU was incorporated into proteoliposomes for bilayer experiments as described previously<sup>14</sup>. *In vitro* expression of pIVEX2.3d:MICU2 was performed using the RTS 100 *E.coli* Disulfide Kit (Roche).

### ***Immunofluorescence***

HeLa cells were grown on 13 mm coverslips and transfected with MICU1-HA or MICU2-Flag encoding plasmid when 50% confluent. After 24 hours, cells were washed with PBS, fixed in 4% formaldehyde for 10 minutes and quenched with 50 mM NH<sub>4</sub>Cl in PBS. Cells were permeabilized for 10 minutes with 0.1% Triton X-100 in PBS and blocked in PBS containing 2% BSA and for 1 hour. Cells were then incubated with primary antibodies ( $\alpha$ TOM20,  $\alpha$ Flag and  $\alpha$ HA) for 3 hours at room temperature and washed 3 times with 0.1% Triton X-100 in PBS. The appropriate isotype matched, AlexaFluor488 or AlexaFluor555 conjugated secondary antibodies (Life Technologies) were used and coverslips were mounted with ProLong Gold Antifade reagent (Life Technologies). Images were taken on a Leica TCS-SP5-II equipped with a 100x, 1.4N.A. Plan-apochromat objective. AlexaFluor488 was excited by the 488 nm laser

lines and images were collected in the 495-535 nm range. AlexaFluor555 was sequentially excited with the 543 nm laser line and signal were collected in the 555-600 nm range. Pixel size was set below 80 nm to meet the Nyquist criterion. For each cell, a z-stack of the whole cell was taken, with a step size of 130 nm. Images are presented as maximum projections of the whole stack.

### ***Förster Resonance Energy Transfer (FRET)***

HeLa cells were grown on 24 mm coverslips and transfected with the indicated combination of GFP, MCU-GFP, MICU1-GFP, MICU2-GFP, mCherry, MCU-mCherry, MICU1-mCherry or MICU2-mCherry encoding plasmids when 50% confluent. GFP and mCherry were excited at 488 and 543 nm and their signals were collected in the 495-535 and 598-670 nm range respectively. A specific region of the specimen was bleached with a 1.5W 592 nm laser (4 passes at 30% power). Donor and acceptor images were collected before and after bleaching, and FRET efficiency was calculated from the background subtracted images with the formula:

$$\text{FRET} = \left( \frac{\text{Donor}_{\text{Post}} - \text{Donor}_{\text{Pre}}}{\text{Donor}_{\text{Post}}} \right) \times 100$$

were  $\text{Donor}_{\text{Pre}}$  and  $\text{Donor}_{\text{Post}}$  are the mean fluorescence intensities in the selected region before and after bleaching. As internal control, no significant FRET was observed outside the bleached region. When small FRET changes were observed outside the bleached region (most likely due to normal organelle dynamics in living cells), the apparent FRET efficiency was subtracted from the whole image. No bleaching of the donor was observed with this experimental settings. Pinhole was set to 1 airy unit and pixel size was adjusted to 150 nm. All images were taken on a Leica TCS STED CW system equipped with a 100x/1.4 N.A. Plan Achromat objective.

### ***Mitochondrial morphology analysis***

Images for mitochondrial morphology were taken on a Leica TCS-SP5-II equipped with a PlanApo 100x/1.4 N.A. objective. For all images, pinhole was set to 1 airy unit, pixel size was 75 nm and a Z-stack was acquired for the whole depth of the cell by sampling at 130 nm in the Z plane in order to meet the Nyquist criterion. Mitochondrial-targeted DsRed was excited by the 543 nm HeNe laser and its emission was collected in the 555-700 nm range. For each image, laser intensity was slightly adjusted in order to maximize signal and avoid saturation. Images were then all analyzed with the Fiji image processing package based on ImageJ (Schindelin et al., 2012). Individual stacks were segmented using a Brensen-based automatic local thresholding algorithm and mitochondrial morphometric parameters (number of object and mean object volume) was calculated with the 3D Object Counter plugin. Representative images are shown as maximum projection of the whole stack.

### ***Mitochondria fractionation and proteinase K protection assay***

Mitochondria were extracted from CD1 mice livers through differential centrifugation as previously described (Frezza et al., 2007). Mitoplasts were obtained through osmotic swelling by incubating

mitochondrial fraction in 20 mM TRIS-Cl pH 7.4 for 20 minutes. The same amount of mitoplasts was treated with proteinase K (100  $\mu\text{g}/\text{mL}$ ) at 4°C for the indicated time and the proteolytic reaction was quenched by PMSF addition. Samples was then loaded on SDS-PAGE and processed for Western blot as described above.

### ***Statistical analysis of data***

Statistical data are presented as mean  $\pm$  S.D. unless specified, significance was calculated by Student's t test, and correlation analysis was performed with the SigmaPlot 12.0 software (Systat Software Inc.) or Excel (Microsoft).

## Supplemental references

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