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Supplemental Information

Downregulation of the Mitochondrial Calcium Uniporter by Cancer-Related miR-25

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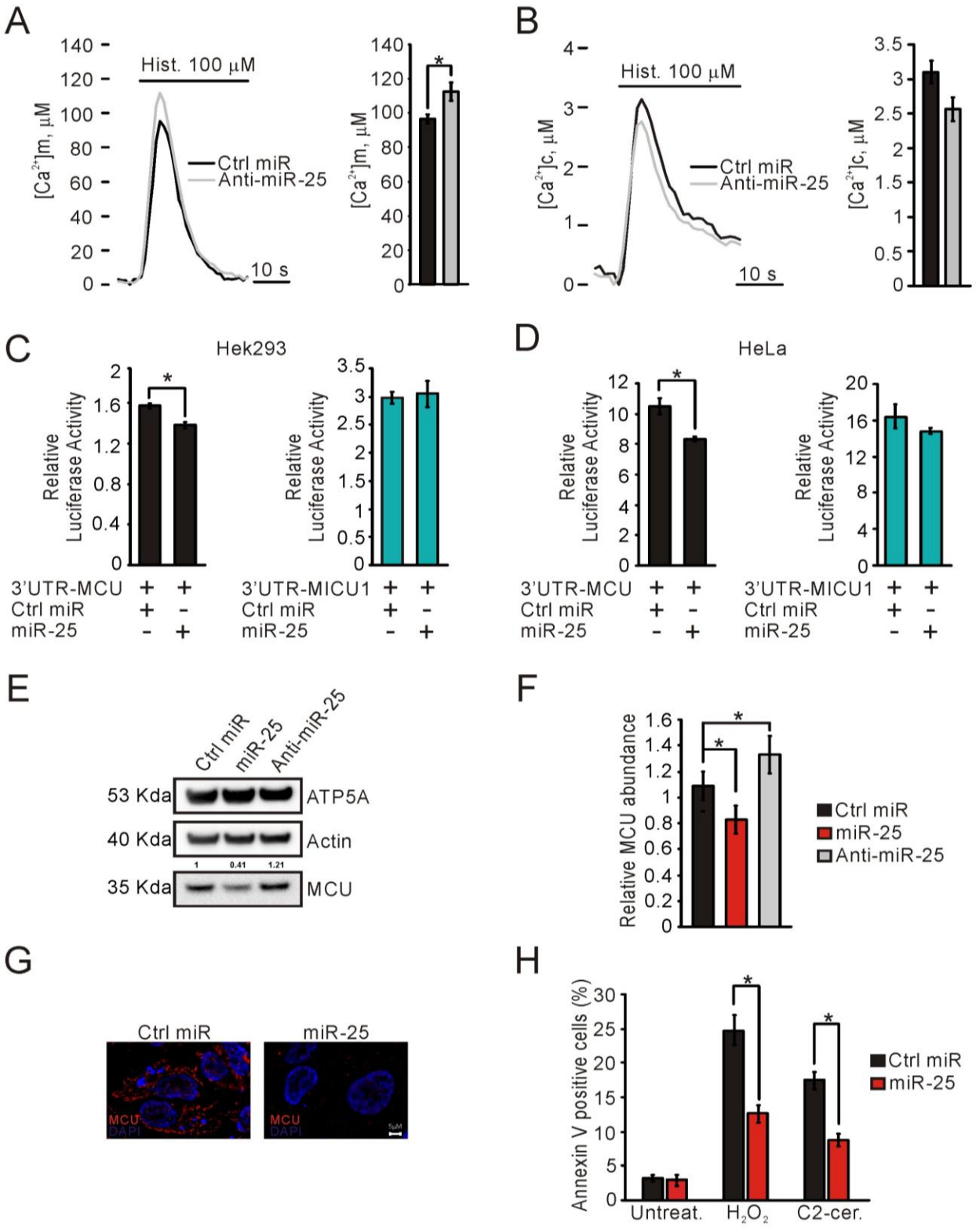
Author Contributions

S. Marchi conceived and performed all experiments, collected and analyzed experimental data, and prepared the manuscript. S.P., A.R., S. Missiroli, A.B., E.D.M., F.P. and C.G. assisted with cell culture and performed some experiments. M.B. assisted with imaging-based experiments. L.L. and F.C. performed quantitative RT-PCR experiments. R.G. and G.L. performed immunohistochemistry experiments and reviewed all experimental data. R.R. and M.N. reviewed all experimental data, and prepared the manuscript. P.P. conceived all experiments, reviewed all experimental data, and prepared the manuscript. All authors discussed the results and reviewed the manuscript.

Supplemental Inventory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C
Hek293
Relative Luciferase Activity
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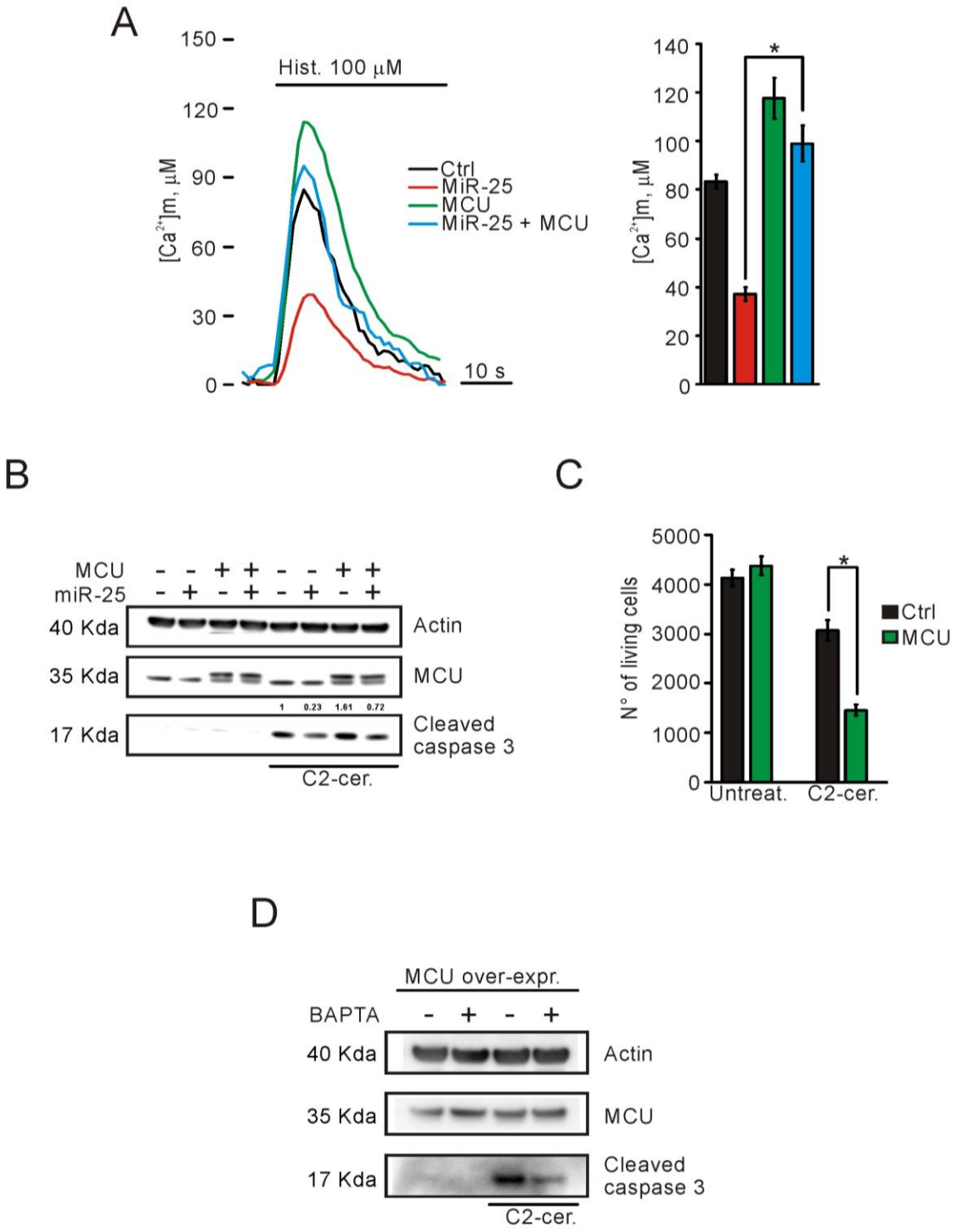
3'UTR-MICU1
Ctrl miR
miR-25
D
HeLa
E
Ctrl miR
miR-25
Anti-miR-25
53 Kda
ATP5A
40 Kda
Actin
35 Kda
MCU
F
Relative MCU abundance
Ctrl miR
miR-25
Anti-miR-25
G
Ctrl miR
miR-25
MCU
DAPI
H
Annexin V positive cells (%)
Ctrl miR
miR-25
Untreat.
H2O2
C2-cer.

Figure S1.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MCU
MiR-25 + MCU
10 s
*
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miR-25
40 Kda
Actin
35 Kda
MCU
17 Kda
Cleaved caspase 3
1
0.23
1.61
0.72
C2-cer.
C
N° of living cells
Ctrl
MCU
Untreat.
C2-cer.
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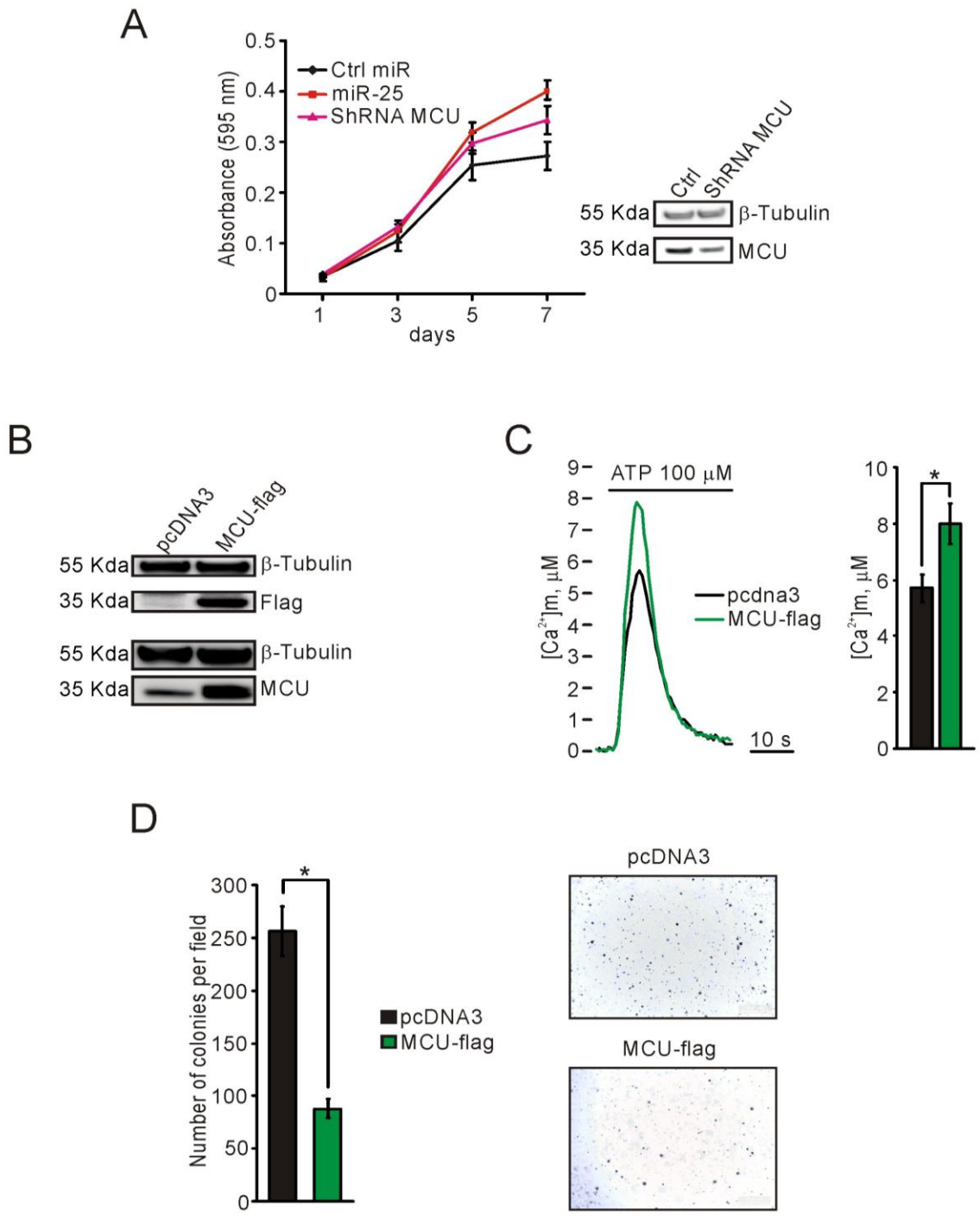
C2-cer.

Figure S2.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0.2
0.1
0
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miR-25
ShRNA MCU
1
3
5
7
days
Ctrl
ShRNA MCU
55 Kda
β-Tubulin
35 Kda
MCU
B
pcDNA3
MCU-flag
55 Kda
β-Tubulin
35 Kda
Flag
55 Kda
β-Tubulin
35 Kda
MCU
C
ATP 100 μM
[Ca2+]m, μM
pcdna3
MCU-flag
10 s
*
D
Number of colonies per field
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250
200
150
100
50
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*
pcDNA3
MCU-flag
pcDNA3
MCU-flag

Figure S3.

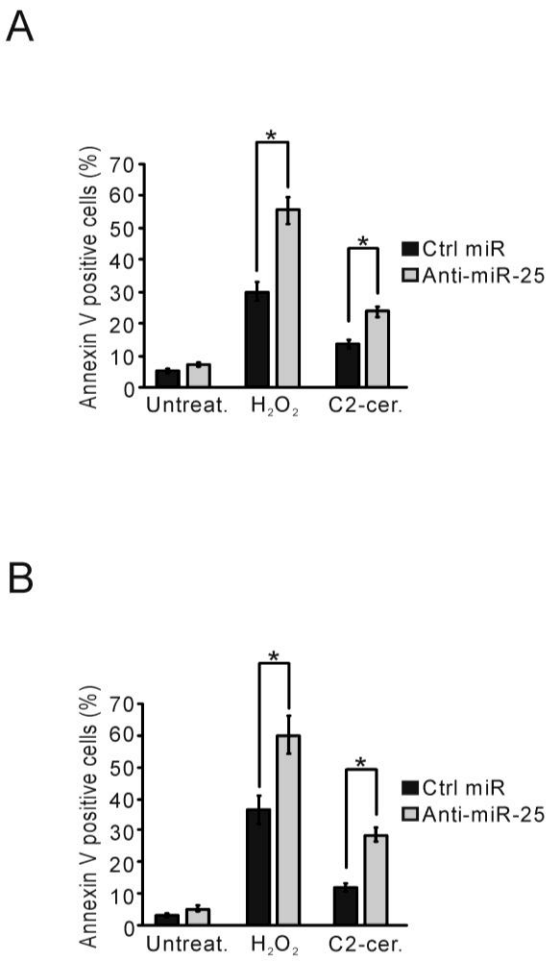

Figure S4.

Table S1.

		miRNA prediction						
miRNA	Target	Tot.	Targetscan	microT	microCosm	Miranda	Cancer Link	
miR-15/16/195/424/497	MCU/MICU1	2/4	X X	X X	X	X	Yes	Down
miR-17-5p/20/93.mr/106/519.d	MCU	1	X				Yes	Up
miR-21/590-5p	MCU	3	X		X	X	Yes	Up
miR-25/32/92/92ab/363/367	MCU/MICU1	3/3	X X	X	X	X X	Yes	Up
miR-137	MCU	4	X	X	X	X	Yes	Down

Table 1 lists some microRNAs involved in human cancer, which target MCU and MICU1 messengers RNA. For each group are indicated which algorithms (Targetscan, microT, microCosm, Miranda) were able to predict the targeting and if these microRNAs were found up or downregulated in human cancer.

Supplemental Figure Legends

Figure S1. Anti-miR-25 Expression Increases MCU Levels and Mitochondrial Ca^{2+} Uptake in HeLa Cells, Related to Figure 1

(**A**) Where indicated mitochondrially targeted aequorin (mtAEQmut)–transfected cells were treated with 100 μM Histamine (Hist.). Mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) peaks: Negative Control (Ctrl mir): 96.63 ± 2.71 μM; Anti-miR-25: 112.58 ± 5.41 μM.

(**B**) Cytosolic Ca^{2+} concentration peaks: Ctrl miR: 3.10 ± 0.167 μM; Anti-miR-25: 2.56 ± 0.172 μM.

(**C-D**) Luciferase assays were performed in Hek293 and HeLa cells. psiCHECK-3'UTR-MCU or psiCHECK-3'UTR-MICU1 constructs were co-transfected with miR-25 or Ctrl miR. Renilla luciferase activity was normalized on firefly luciferase activity. Relative luciferase activity of psiCHECK-3'UTR-MCU displayed 12% and 20% decrease following miR-25 enforced expression if compared to negative control, respectively in Hek293 (**C**) and HeLa (**D**) cells. No significant differences were found in relative luciferase activity of psiCHECK-3'UTR-MICU1 construct in both cell lines.

(**E**) Immunoblot analysis of MCU protein after miR-25 and anti-miR-25 expression in HeLa cells. ATP5A has been used as inner mitochondrial membrane marker. Quantification of MCU protein is reported.

(**F**) MCU mRNA expression was assessed through quantitative Real Time PCR in HeLa cells transfected with miR-25, anti-miR-25 or Ctrl miR. GAPDH expression was used to normalize MCU expression results for each sample. MiR-25 enforced expression caused a 30% decrease in MCU mRNA levels, whereas anti-miR-25 transfection induced a 28% increase in MCU expression, if compared to control transfected cells.

(**G**) Immunofluorescence showing MCU down-regulation by miR-25.

(**H**) HeLa cells were treated with Hydrogen Peroxide (H_2O_2; 500 μM for 2 h.) or C2-ceramide (C2-cer.; 40 μM for 2 h.), and subsequently stained with annexin V-Alexa fluor 488.

Error bars correspond to mean ± SEM of at least three independent experiments. *$p < 0.05$.

Figure S2. MCU Reintroduction Restores miR-25 Activity, Related to Figure 2

(**A**) Mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) peaks: Negative Control (Ctrl miR): 83.36 ± 2.656 μM; mir-25: 39.04 ± 2.875 μM; MCU: 118.63 ± 8.5 μM; mir-25 + MCU: 99.06 ± 7.4 μM.

(**B**) Immunoblot analysis showing cleaved caspase 3 levels, after treatment with C2-cer (40 μM for 2 h.). Quantification of cleaved caspase 3 protein is reported.

(**C**) Microscopy counts of cell viability after treatment with C2-ceramide (40 μM for 2 h.), in 22Rv1 cells, after MCU over-expression.

(**D**) Immunoblot analysis of MCU-overexpressing HeLa cells, showing cleaved caspase 3 levels, after treatment with C2-cer (40 μM for 2 h.). Where indicated, cells were pre-treated (5 μM, 20 min) with the intracellular Ca^{2+} buffer BAPTA-AM (Invitrogen).

Error bars correspond to mean ± SEM of at least three independent experiments. *$p < 0.05$.

Figure S3. miR-25 Proto-oncogenic Activity, Related to Figure 3

(**A**) Growth curve of HeLa cells after miR-25 or ShRNA MCU expression. Immunoblot analysis on the right.

(**B**) Immunoblot analysis of PC3 cells stably expressing pcDNA3 empty vector (pcDNA3) or MCU-flag tagged, in pcDNA3 vector (MCU-flag).

(**C**) Mitochondrial Ca^{2+} concentration ($[Ca^{2+}]_m$) peaks: pcDNA3: 5.7 ± 0.47 μM; MCU-flag: 8 ± 0.71 μM.

(**D**) Number of colonies formed in soft agar; representative fields on the right.

Error bars correspond to mean ± SEM of at least three independent experiments. *$p < 0.05$.

Figure S4. Anti-miR-25 Sensitizes PC3 and HCT116 Cells to Apoptosis, Related to Figure 4

PC3 (**A**) and HCT116 (**B**) cells were treated with Hydrogen Peroxide (H_2O_2; 500 μM for 2 h.) or C2-ceramide (C2-cer.; 40 μM for 2 h.), and subsequently stained with annexin V-Alexa fluor 488. Percentage of Annexin V-positive cells is shown. Error bars correspond to mean ± SEM of three independent experiments. *$p < 0.05$.

Supplemental Experimental Procedures

Vectors and Luciferase Assay

Portions of 3'UTR of human MCU and MICU1 genes, containing miR-25 putative target regions, were amplified through PCR, using the following primers:
MCU_3UTR_F: 5'-CACTCGAGACACTGCATGAGGTTGTTGG-3'
MCU_3UTR_R: 5'-CAGTTTAAACCACCTGGAGTCTGGGTTTGT-3' (760 bp);
MICU1_3UTR_F: 5'-CACTCGAGAGAATTCAGGGAACCATCCA-3'
MICU1_3UTR_R: 5'-CAGTTTAAACACAGGGAACTTTGGGGATGT-3' (570 bp).
These regions were cloned into psiCHECK-2 vector (Promega), downstream of renilla luciferase gene, using XhoI and PmeI restriction sites. For luciferase assay Hek-293 and Hela cells were cultured in 24-well plate and transfected in triplicate with 400 ng of psiCHECK-3'UTR-MCU, psiCHECK-3'UTR-MICU1 constructs or psiCHECK control vector and 50 pmol of miR-25 or Negative Control 2 (Ambion). Transfection was performed using Lipofectamine 2000 and Optimem I Reduced Serum Medium (Invitrogen), as described by the manufacturer. Luciferase activity measurement was performed 24 hours after transfection, using Dual-Luciferase Reporter Assay (Promega), following the protocol of the kit. Activity of firefly luciferase was used to normalize renilla luciferase activity, for each well.

Real-Time RT-PCR to Evaluate miRNA and mRNA Expression

Total RNA was extracted from cells with Trizol reagent (Invitrogen), according to the manufacturer's instructions. Mature miR-25 expression was assessed using TaqMan microRNA assay – has-miR-25-3p (Applied Biosystem – 000403) and normalized on RNU6B (Applied Biosystem – 001093). Five ng of total RNA were reverse-transcribed using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystem) and the looped primer provided by the specific TaqMan microRNA assay. The quantitative PCR reaction mix was prepared using TaqMan Universal PCR Master Mix, No Amperase UNG (Applied Biosystem) and specific TaqMan primers and probe provided by TaqMan microRNA assay kits. Reactions were carried out in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, on Bio-Rad-Chromo4 Real Time thermal Cycler. Each sample was analyzed in triplicate. The level of miRNA was measured using Ct (threshold cycle) and to calculate the amount of miRNA, the method of 2-ΔΔCt was used. To analyze mRNA expression, qRT-PCR was performed on 500 ng of total RNA using oligo dT (Fermentas) and random primers (Gibco). Quantitative PCR reaction was performed using Qiagen Taq DNA Polymerase (Qiagen) and EvaGreen (Biotium Inc). The following oligonucleotides were used as primers for the qPCR reaction:
MCU_RT_F: 5'-TTCCTGGCAGAATTTGGGAG-3'
MCU_RT_R: 5'- AGAGATAGGCTTGAGTGTGAAC-3'
GAPDH_RT_F: 5'-CTATAAATTGAGCCCGCAGCC-3'
GAPDH_RT_R: 5'-CCCAATACGACCAAATCCGT-3'
18S_RT_F: 5'- CTGCCCTATCAACTTTCGATGGTAG-3'
18S_RT_R: 5'-CCGTTTCTCAGGCTCCCTCTC-3'.
Reactions were incubated in a 96-well PCR plate at 95°C for 15 min, followed by 40 cycles of 95° C for 30 sec and 58°C for 1 min, on Bio-Rad-Chromo4 Real Time thermal Cycler. Each sample was analyzed in triplicate. 18S RNA expressions were used as endogenous reference control. The level of mRNA was measured using Ct (threshold cycle) and to calculate the amount of mRNA, the method of 2-ΔΔCt was used.

Aequorin Measurements

Probes employed are chimeric aequorins targeted to the endoplasmic reticulum (erAEQmut), cytosol (cytAEQ), and mitochondria (mtAEQmut). "AEQ" refers to wild-type aequorin, and "AEQmut" refers to a low affinity D119A mutant of aequorin. For the experiments with cytAEQ, mtAEQ and mtAEQmut, cells were incubated with 5 μM coelenterazine for 1–2 h in Dulbecco's modified Eagle's medium supplemented with 1% fetal calf serum. A coverslip with transfected cells was placed in a perfused thermostated chamber located in close proximity to a low noise photomultiplier with a built-in amplifier/discriminator. To reconstitute erAEQmut with high efficiency, the luminal [Ca^{2+}] of the ER first had to be reduced. This was achieved by incubating cells for 1 h at 4°C in Krebs-Ringer buffer (KRB) supplemented with 5 μM coelenterazine, 5 μM Ca^{2+} ionophore ionomycin (Sigma-Aldrich), and 600 μM EGTA. After this incubation, cells were extensively washed with KRB supplemented with 2% bovine serum albumin and then transferred to the perfusion chamber. All aequorin measurements were carried out in KRB supplemented with either 1 mM $CaCl_2$ (cytAEQ and mtAEQmut) or 100 μM EGTA (erAEQmut). Agonist was added to the same medium as specified in figure legends. The experiments were terminated by lysing cells with 100 μM digitonin in a hypotonic Ca^{2+}-containing solution (10 mM $CaCl_2$ in H_2O), thus discharging the remaining aequorin pool. The output of the discriminator was captured by a Thorn EMI photon-counting board and stored in an IBM-compatible computer for further analyses. The aequorin luminescence data were calibrated offline into [Ca^{2+}] values using a computer algorithm based on the Ca^{2+} response curve of wild-type and mutant aequorins.

In the experiments with permeabilized cells, a buffer mimicking the cytosolic ionic composition, (intracellular buffer) was used: 130 mM KCl, 10 mM NaCl, 2 mM K_2HPO_4, 5 mM succinic acid, 5 mM malic acid, 1 mM $MgCl_2$, 20 mM HEPES, 1 mM pyruvate, 0.5 mM ATP and 0.1 mM ADP (pH 7 at 37 °C). Intracellular buffer was supplemented with either 100 μM EGTA (intracellular buffer/EGTA) or a 2 mM EGTA and 2 mM HEEDTA-buffered [Ca^{2+}] of 1 or 4 μM (intracellular buffer/Ca^{2+}), calculated with the Chelator software [27]. HeLa cells were permeabilized by a 1-min perfusion with 50 μM digitonin (added to intracellular buffer/EGTA) during luminescence measurements. Mitochondrial Ca^{2+} uptake rate was calculated as the first derivative by using the OriginLab® software. The higher value reached during Ca^{2+} addition represents the maximal Ca^{2+} uptake rate.

Mitochondrial Membrane Potential (ΔΨm) Measurements

HeLa cells were seeded and transfected with the indicated miR. MiR expression was allowed for 24 hours and mitochondrial ΔΨ was measured by loading cells with 20 nM tetramethyl rhodamine methyl ester (TMRM, Invitrogen) for 30 min at 37 °C. Successively, cells where imaged with Nikon Swept Field Confocal equipped with CFI Plan Apo VC60XH objective (n.a. 1.4) and an Andor DU885 EM-CCD camera, controlled by the NIS-Elements 3.2. Basal levels were normalized on fluorescence in presence of FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone, 10 μM), a strong uncoupler of oxidative phosphorylation.

Imaging and Analysis of Mitochondrial Morphology

HeLa cells were seeded on 24-mm coverslips and transfected using Lipofectamine 2000 as previously described. During transfection control miR or miR-25 were cotransfected with mt-DsRed and ErGFP.

At 24 hours after transfection, cells were imaged with a laser scanning confocal Zeiss LSM 510, illuminating GFP at 488 nm and dsRed at 543 nm. Z stack of 51 planes were obtained with an objective Plan-Apo 63x/1.4 Oil Ph3 with a voxel size of 105 x 105 x 200 nm (X x Y x Z). To obtain the best object reality, images were next deconvolved using the open source software Fiji (http://fiji.sc/wiki/index.php/Fiji, last accessed June 20, 2011), and especially through the 3D iterative deconvolution plugin (http://www.optinav.com/Iterative-Deconvolve-3D.htm). A theoretical PSF were build using the "PSF generator" plugin available at http://bigwww.epfl.ch/algorithms/psfgenerator/.

Once reconstructed a mitochondrial and endoplasmic reticulum mask were manually chosen to obtain a binarized image of overlapping areas. The resulting areas were described in number and volume, using the 3D object counter, available in Fiji.

Immunoblotting

Total cell lysates were prepared in RIPA buffer and the standard immunoblotting procedure was used. Proteins were quantified by the bicinchoninic acid assay (BCA) method and 20 μg of each sample were loaded on a Novex NuPage Bis-Tris 4–12% precast gel (Invitrogen) and transferred onto nitrocellulose membranes. Isotype-matched, horseradish-peroxidase-conjugated secondary antibodies (Santa Cruz Biotech.) were used, followed by detection by chemiluminescence (ThermoScientific), using ImageQuant LAS 4000 (GE Healthcare).

Primary antibodies used were: rabbit anti-PARP and rabbit anti-Caspase 3 from Cell Signalling; rabbit anti-Actin, rabbit anti-MCU, rabbit anti-Flag and mouse anti-β tubulin from Sigma-Aldrich; rabbit anti-MICU1 and rabbit anti-ATP5A from Abcam.

Immunohistochemistry

Four-micrometer thick sections were cut from formalin-fixed paraffin-embedded blocks. One section for each block was routinely stained with hematoxylin and eosin for histological examination.

For immunodetection of MCU, tissue sections were deparaffinized with xilene and rehydrated by sequential ethanol (from 100% to 80%) and rinsed in distilled water. Before immunostaining, sections were processed by microwave-oven for antigen retrival in Tris-EDTA-Citrate buffer (pH 7.8) for 30 minutes. After rinse with distilled water and rehydratation with PBS buffer, sections were incubated in a buffer solution with 3% of H_2O_2 for 15 minutes at room temperature to block endogenous peroxidase activity.

Tissue sections were then incubated with the primary rabbit anti-MCU antibody (Sigma-Aldrich), diluted 1:100 for 1h at room temperature. We then used the Ultravision Detection System (Large Volume Polyvalent-HRP) (Thermo Scientific) and the Dab Detection Kit (Cell Marque) according to manufacturers' instructions. Counterstaining was conducted with Mayer's hematoxylin.

Immunofluorescence

Cells were fixed with 4% formaldehyde for 10 min at RT. After washing three times with phosphate-buffered saline (PBS), cells were permeabilized with 0.1% Triton X-100 in PBS (PBST) at RT for 10 min and treated with PBST containing 5% skim milk (PBSTM) at RT for 1 h. Cells were incubated with antibody to MCU in PBSTM overnight at 4 °C, washed three times with PBS, and then incubated with Alexa-594-conjugated anti-rabbit IgG (Molecular Probes) at RT for 1 h.

Images were acquired through an epifluorescent microscope Axiovert 200M (ZEISS) equipped with a 100x Pla-Neofluar n.a. 1.3 (Zeiss) and a CoolSnap HQ (Photometrics). Each field was acquired as z-stack (21 planes spaced by 0.5 μM) then deconvolved through the open source software Fiji (available at http://fiji.sc/) and the parallel iterative deconvolution plugin.

Cell Proliferation

8 hours after transfection, the cells of one 6-well dish were trypsinized, counted with Burker chamber, and plated in four sets of five wells of a 12-well plate. Starting from the following day (day 1), 1 set of wells (at days 3, 5 and 7) was washed once with PBS, fixed in 4% formaldehyde solution for 10 min at room temperature, and then kept in PBS at 4°C. At day 7, all the wells were stained with crystal violet. After lysis with 10% acetic acid, the absorbance was read at 595 nm.

Growth in Semisolid Medium

The bottom layer was obtained by covering six-well dishes with 3 ml of 0.6% agar in RPMI. The following day, 5×10^4 stable clone PC3 cells were plated on this bottom layer in triplicate, in 2 ml of 0.3% agar in RPMI + 10% FBS. After 4 weeks, colonies were stained with 0.005% crystal violet and counted at 4x magnification. Five fields for each well were counted. A Leica DM IL LED microscope was used. Colonies were counted automatically using a custom made macro in the Fiji software. Briefly, dark objects were thresholded using the Yen algorithm and counted through the analyze particles tool; objects smaller than 70 pixels were excluded.

Measurement of Annexin V Binding

Cells were centrifuged, washed with PBS and the cell pellet was resuspended in 100 μl of labelling solution, containing 5 μl annexin V Alexa fluor 488 reagent (Invitrogen). After 20 min incubation, according to manufacturer's instructions, cells were measured at Tali™ image-based cytometer (Invitrogen). Percentage of positive cells has been reported.

Statistical Analysis

In each graph, unless noted, data represent mean ± SEM. If indicated, statistical significance has been calculated by a two-tailed Student t-test between the indicated samples. p values are indicated in the legends.

Supplemental References

27. Schoenmakers, T.J., Visser, G.J., Flik, G., and Theuvenet, A.P. (1992). CHELATOR: an improved method for computing metal ion concentrations in physiological solutions. BioTechniques *12*, 870-874, 876-879.