Supporting Information
Supplementary Methods
Cells
MICU1-KO (M1KO) and corresponding WT MEFs were grown as previously described [1].
Acute transfection of pcDNA3-dest40, mito-mRFP-T2A-MICU1-HA and mito-mRFP-T2A-MICU1-HA-mEF1-mEF2 (kindly provided by Peter Varnai) was performed in HEKs and MEFs using Lipofectamine 3000 according to manufacturers' protocol.

Measurements of $^{45}Ca^{2+}$ uptake by mitochondria in permeabilized MEFs
For these experiments, transfected cells were sorted based on the RFP fluorescence on the Thomas Jefferson University platform of flow cytometry (BD FACS Melody streamline cell sorter). Cells were resuspended in ICM containing 2 μM Tg, 20 μM CGP-37157, and 10 μM EGTA. The cells were permeabilized by saponin (40 μg/ml) in the presence of 1 mM succinate and 2 mM MgATP for 5 min at 37˚C with stirring. Subsequently, the suspensions were mixed with different amounts of ^{45}Ca in the absence or presence of Ruthenium Red (3 μM). At 30s time points, 100μl aliquots were stopped and ^{45}Ca in the mitochondria was quantified as described [2]. Free [Ca^{2+}] in extramitochondrial medium was measured separately by fluorometric measurements using fura-2 or furaFF.

Immunoblot analysis
Protein lysates from cells were prepared as described in the corresponding main method section. Equivalent amounts of total protein (50 μg) were separated electrophoretically by SDS-PAGE (10%, Biorad) in reducing conditions and transferred to a nitrocellulose membrane (Biorad). Further steps were similar to those described in the corresponding main method section. EMRE (Santa Cruz, sc-86337, 1:200) was used.

Permeabilized cell imaging
Experiments were performed as described in the corresponding main method section. In some of the experiment (FigS3), EDTA addition was followed by addition of a channel-forming ionophore, alamethicin (2.5 μM). In several experiments (FigS6), prior to the permeabilization the transfected cells were identified by mito-mRFP (550 nm excitation) that was simultaneously expressed with MICU1-HA and MICU1-HA-mEF1-mEF2 in bicistronic constructs.

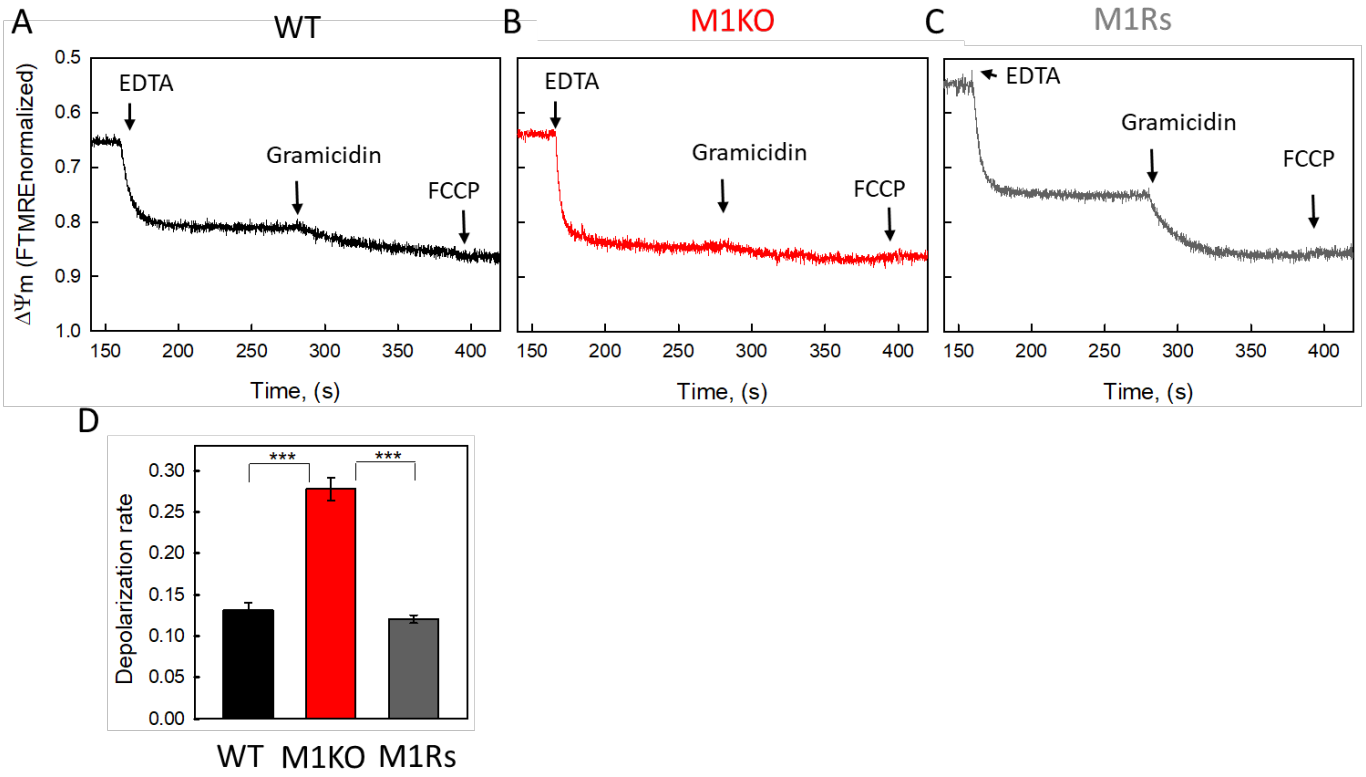
Statistical analysis
Data are expressed as MEAN ± SEM. For FigS7B-D, paired t-test was used. For FigS6 one-way ANOVA with Dunn's post-hoc test was used. *$p < 0.05$.

References

1. Paillard, M., et al., *MICU1 Interacts with the D-Ring of the MCU Pore to Control Its Ca(2+) Flux and Sensitivity to Ru360.* Mol Cell, 2018. **72**(4): p. 778-785 e3.
2. Csordas, G., et al., *MICU1 controls both the threshold and cooperative activation of the mitochondrial Ca(2)(+) uniporter.* Cell Metab, 2013. **17**(6): p. 976-987.

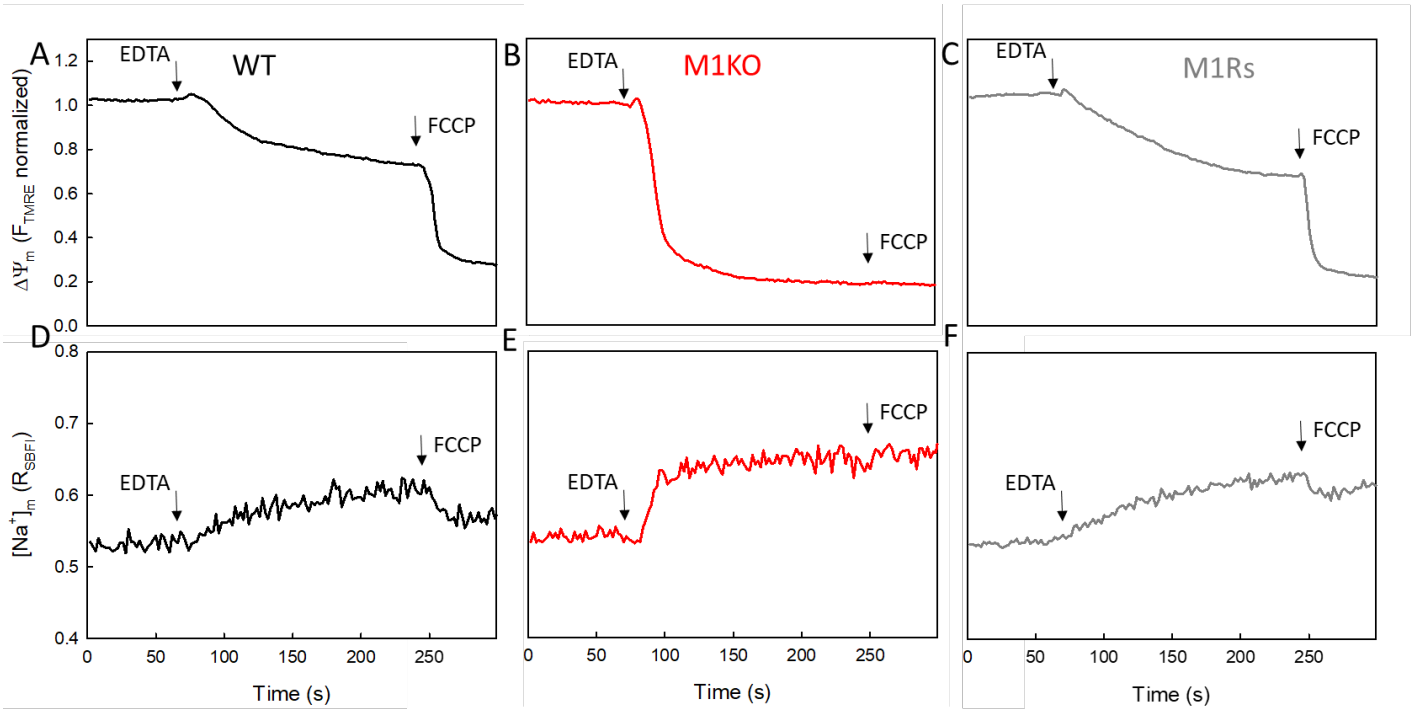
Supplementary Figures

Fig. S1



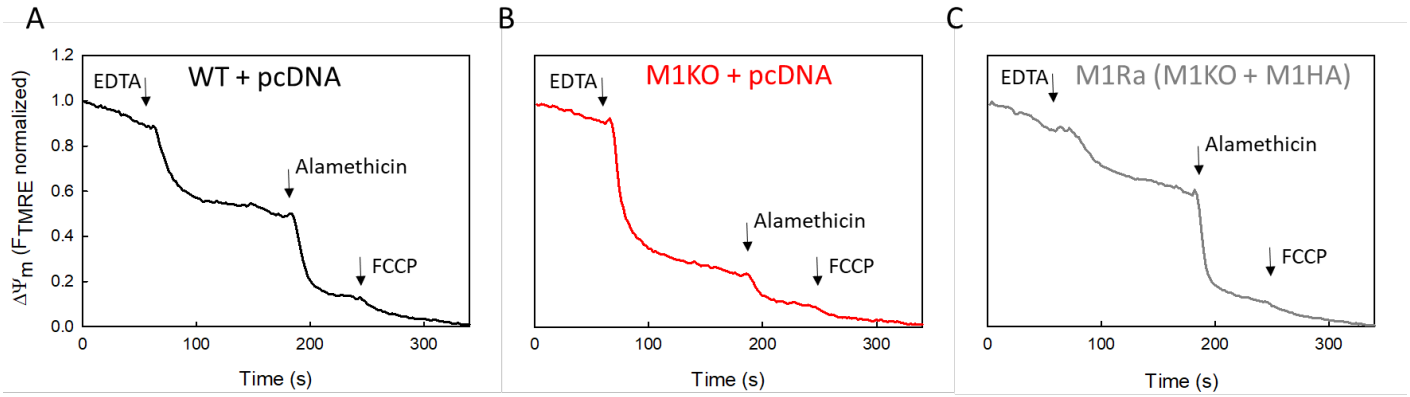
FigS1. MICU1 controls the Na^+ influx-induced depolarization (A-C) in permeabilized HEK cells. Na^+ influx into mitochondria was induced by depleting Mg^{2+} and Ca^{2+} with EDTA (0.5 mM). Gramicidin (1 μM) was added after to maximize the Na^+ influx. In the end, an uncoupler, FCCP (2 μM), was added to attain complete dissipation of $\Delta\Psi m$. EDTA-induced depolarization rate in permeabilized HEK cells in indicated genotypes was estimated (D). Traces (20 s following EDTA addition) were fit to f = y0+a*(1-exp(-b*t)) (R^2 > 0.9) and depolarization rate was calculated as $dF_{normalized}/dt$ (t =0) = a*b and normalized to ΔF(FCCP). Data (N (WT) = 5, N(M1KO) = 8, N (M1Rs) = 9) were compared using one-way ANOVA with Holm-Sidak Post Hoc test. MEAN ± SEM, *** p < 0.001.

Fig. S2



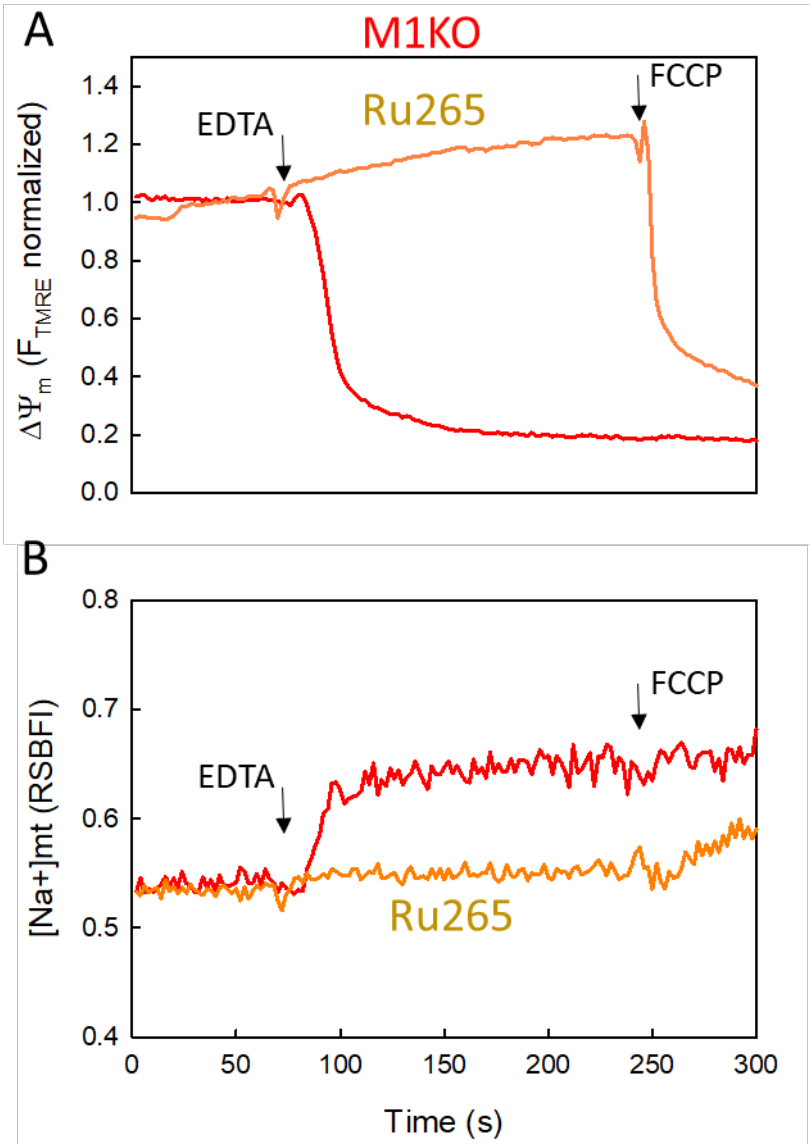
FigS2. MICU1 controls the Na^+ influx-induced $[Na^+]_m$ increase (D-F) and the accompanying depolarization (A-C) in adherent permeabilized HEK cells. Na^+ influx into mitochondria was induced by depleting Mg^{2+} and Ca^{2+} with EDTA (0.5 mM). An uncoupler, FCCP (2 μM), was added in the end to attain complete dissipation of $\Delta\Psi_m$. Traces in (A-C) were normalized to the baseline. Calculation and statistics for the traces are presented in Figs2-3.

Fig. S3



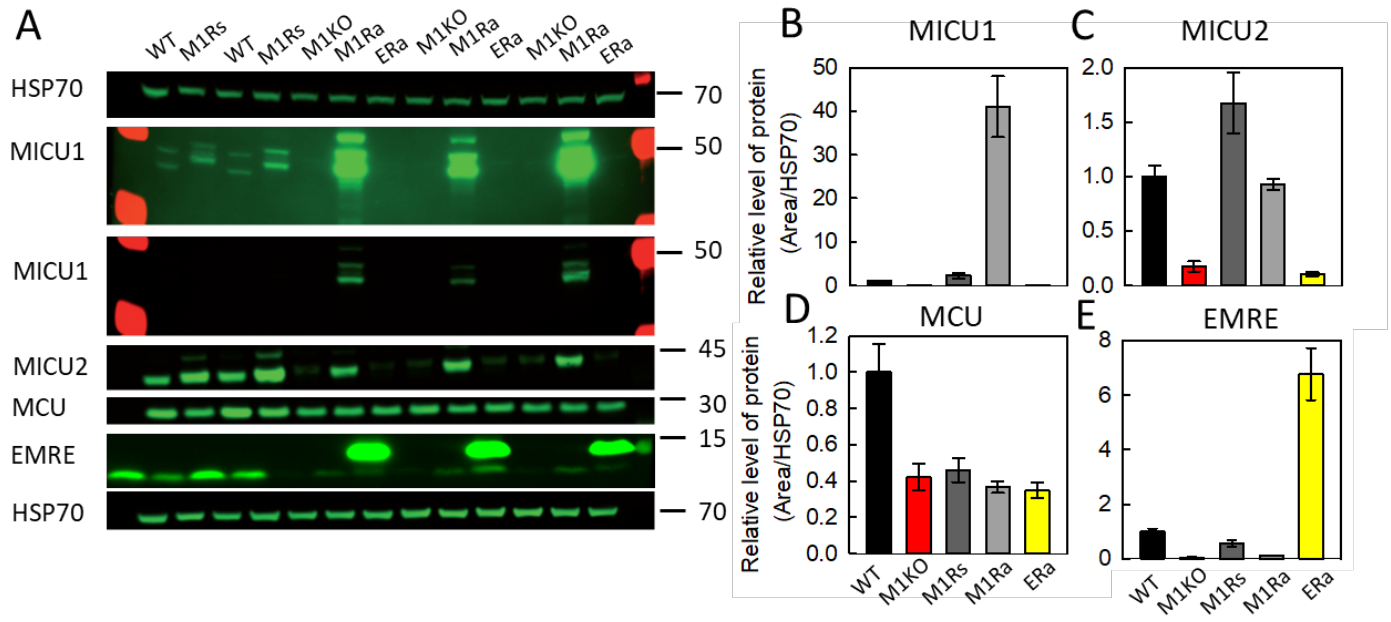
FigS3. Na^+ influx-induced mitochondrial depolarization in divalent-free conditions in permeabilized HEK cells is not altered by transfection itself and can be significantly enhanced by Na^+ entry through a channel-forming ionophore, alamethicin (2.5 μM), in WT (A) and M1Rs (C), but not in M1KO (B). The cells were loaded with TMRE (25 nM) and permeabilized with saponin (25 μg/ml) to measure $\Delta\Psi_m$. Na^+ influx into mitochondria was induced by depleting Mg^{2+} and Ca^{2+} with EDTA (0.5 mM) and further enhanced by addition of alamethicin. An uncoupler, FCCP (2 μM), was added in the end to attain complete dissipation of $\Delta\Psi_m$. WT and M1KO cells were transfected with pcDNA3 to test the effect of transfection itself. N(WT/Ru265) = 29/18, N(M1KO/Ru265) = 16/18, N(M1Rs/Ru265) = 12/19.

Fig. S4



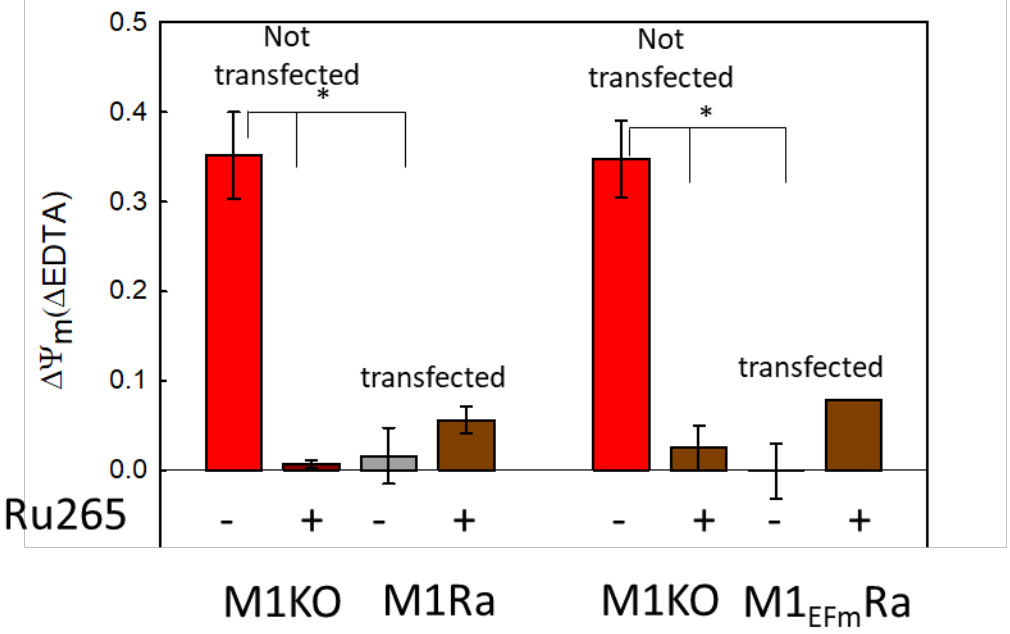
FigS4. Na^+ influx-induced $[Na^+]_m$ increase and accompanying mitochondrial depolarization in adherent permeabilized HEK in divalent-free conditions is Ru265-sensitive. M1KO cells were loaded with TMRE (25 nM) and SBFI AM (10 μM) and permeabilized with saponin (25 μg/ml) to measure $\Delta\Psi_m$ (A) and $[Na^+]_m$ (B). Na^+ influx into mitochondria was induced by depleting Mg^{2+} and Ca^{2+} with EDTA (0.5 mM) in the absence (N = 13) and presence (N = 7) of Ru265 (3 μM). An uncoupler, FCCP (2 μM), was added in the end to attain complete dissipation of $\Delta\Psi_m$.

Fig. S5



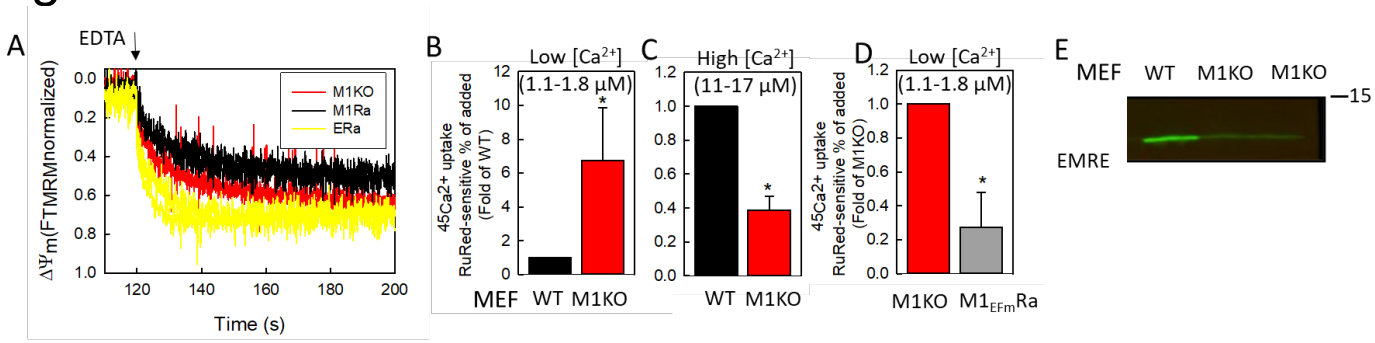
FigS5. Immunoblot analysis of mtCU components in WT, M1KO, M1Rs, M1Ra, and ERa HEK cells performed as described in the main method section (A). MICU1 (B), MICU2 (C), MCU (D), and EMRE (E) levels relative to HSP70 were calculated. MW in A is indicated in kDa. N(WT) = 9, N(M1KO) = 8, N(M1Rs) = 6, N(ERa) = 3, N(M1Ra) = 3. MEAN ± SEM.

Fig. S6



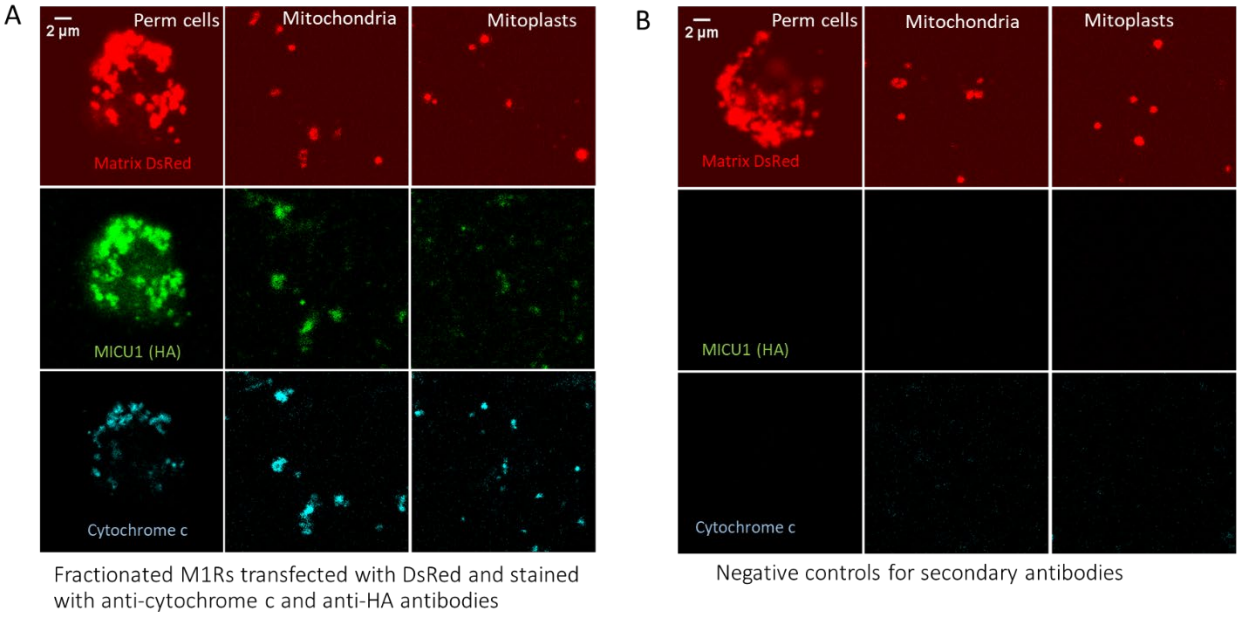
FigS6. Acute overexpression of MICU1 or its EF-hand mutant prevents the mtCU Na^+ influx. EDTA-induced mitochondrial depolarization in divalent-free conditions in the presence and absence of Ru265 (3 μM) is calculated for the indicated genotypes as described in Fig3. M1KO cells were transfected with bicistronic constructs that allowed simultaneous expression of mito-mRFP and MICU1 or $MICU1_{EFm}$. During the experiment, transfected cells were identified prior to permeabilization by fluorescence of mito-mRFP that allowed direct comparison of Na^+ influx-induced mitochondrial depolarization between M1KO and M1Ra or $M1_{EFm}Ra$ in the same imaging field. Data (N(M1KO/Ru265) = 76/27, N(M1Ra/Ru265) = 139/33, N(M1KO/Ru265) = 85/12, N($M1_{EFm}$Ra/Ru265) = 86/20) were compared using one-way ANOVA with Dunn's post-hoc test. MEAN ± SEM, * $p < 0.05$.

Fig. S7



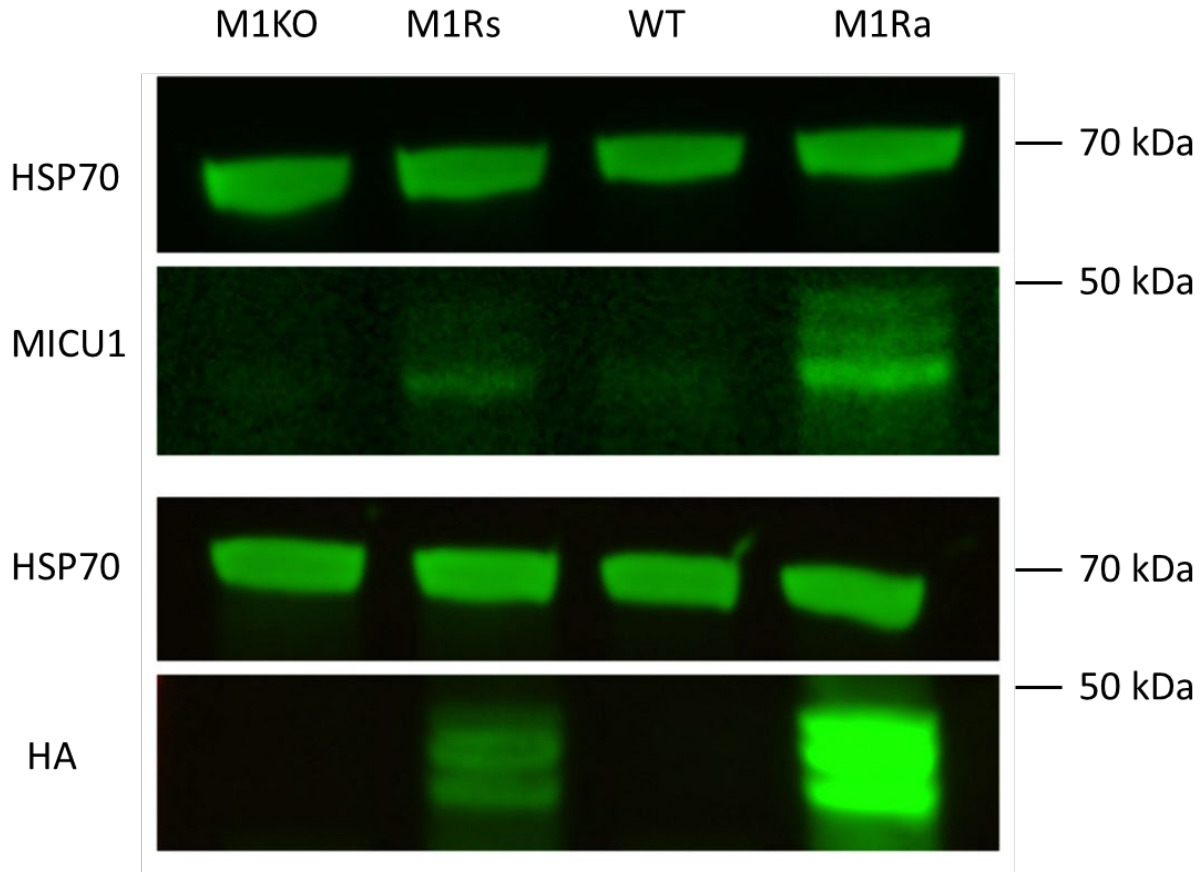
FigS7. Mitochondrial depolarization induced by Na^+ entry following EDTA (0.5 mM) addition in divalent-free conditions in HEK cell suspensions is faster in ERa and reduced in M1Ra (the traces are normalized to the baseline and complete dissipation of $\Delta\Psi_m$ upon FCCP (2 μM)), (N = 2) (A). Mitochondrial Ca^{2+} uptake in permeabilized MEF of the indicated genotypes at the indicated [Ca^{2+}] estimated by Ruthenium Red-sensitive $^{45}Ca^{2+}$ accumulation (B, N = 5; C,D N = 3). Immunoblot analysis of EMRE (performed as described in supplemental methods) in WT and M1KO MEFs (E). MW in E is indicated in kDa. Data are analyzed by paired t-test, MEAN ± SEM. *p < 0.05

Fig. S8



FigS8. M1Rs HEK transfected with mtDsRed were fractionated to permeabilized cells (perm cells), mitochondria, and mitoplasts and further immunostained against anti-HA and anti-cytochrome *c* and imaged (A). Corresponding negative controls with secondary antibody staining omitted (B).

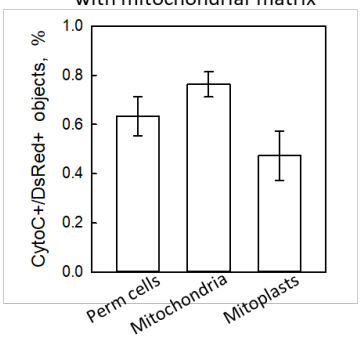
Fig. S9



FigS9. Immunoblot analysis of WT, M1KO, M1Rs, and M1Ra HEK cells against indicated proteins. MICU1-HA staining is specific and present only in M1Rs and M1Ra, but not in WT or M1KO.

Fig. S10

A



B

Permeabilized cells

%	HA + (MICU1)	HA – (MICU1)	
CytoC+	57 ± 10	7 ± 3	64
CytoC-	24 ± 2	12 ± 6	36
	81	19	

Mitochondria

%	HA + (MICU1)	HA – (MICU1)	
CytoC+	64 ± 2	12 ± 4	76
CytoC-	9 ± 4	15 ± 1	24
	73	27	

Mitoplasts

%	HA + (MICU1)	HA – (MICU1)	
CytoC+	18 ± 9	30 ± 2	48
CytoC-	8 ± 1	44 ± 11	52
	26	74	

FigS10. Colocalization of mtDsRed and cytochrome *c* (CytoC) for FigS8A calculated as described in Fig5C (N = 3) (A). Colocalization of cytochrome *c* and HA-tagged MICU1 (B). Data were compared using one-way ANOVA with Holm-Sidak Post Hoc test. MEAN ± SEM.