**Figure S1**

**A**

WT MICU1 KO MICU1 KO + MICU1-FLAG
MICU2 KO MICU2 KO + MICU2-FLAG

**B**

WT cells with:
- none
- 1 μM Ru360
- 1 μM CCCP
- 3 μM Thapsigargin

Oregon Green-Bapta6F (a.u.)

**C**

Rel. Ca\(^{2+}\) Uptake Rate

Fluo-4 (a.u.)

**D**

~1 μM Ca\(^{2+}\)

**E**

~40 μM Ca\(^{2+}\)
Figure S1: Characterization of MICU1 KO and MICU2 KO HEK-293T cells

(A) Immunoblot of whole cell lysates from WT, MICU1 KO, or MICU2 KO HEK-293T cells with or without stable expression of MICU1-FLAG or MICU2-FLAG, using the antibodies indicated on the left, including the loading control SDHB. (B–C) Response of digitonin-permeabilized cells to CaCl₂ pulses, with representative traces shown on the left and quantification from linear fits shown on the right. (B) WT cells with or without 1 μM Ru360, 1 μM CCCP, or 3 μM thapsigargin are exposed to 40 μM CaCl₂ and extramitochondrial Ca²⁺ is monitored with Oregon Green-Bapta6F. (C) MICU1 KO or MICU2 KO cells with or without 1 μM Ru360, 1 μM CCCP, or 3 μM thapsigargin are exposed to ~1 μM CaCl₂ and extramitochondrial Ca²⁺ is monitored with Fluo-4. n.s. denotes not significant. (D–E) Representative traces showing the response of WT, MICU1 KO, MICU1 KO + MICU1-FLAG, MICU2 KO, or MICU2 KO + MICU2-FLAG digitonin-permeabilized cells to a (D) small (~1 μM) or (E) large (~40 μM) pulse of Ca²⁺, monitoring extramitochondrial Ca²⁺ with Fluo-4 or Oregon Green-Bapta6F, respectively. Quantification is included in Figures 1B, D, G.