Cell Reports, Volume *33*

Supplemental Information

Mechanisms of EMRE-Dependent MCU Opening in the Mitochondrial Calcium Uniporter Complex

Anna M. Van Keuren, Chen-Wei Tsai, Enrique Balderas, Madison X. Rodriguez, Dipayan Chaudhuri, and Ming-Feng Tsai

Figure S1

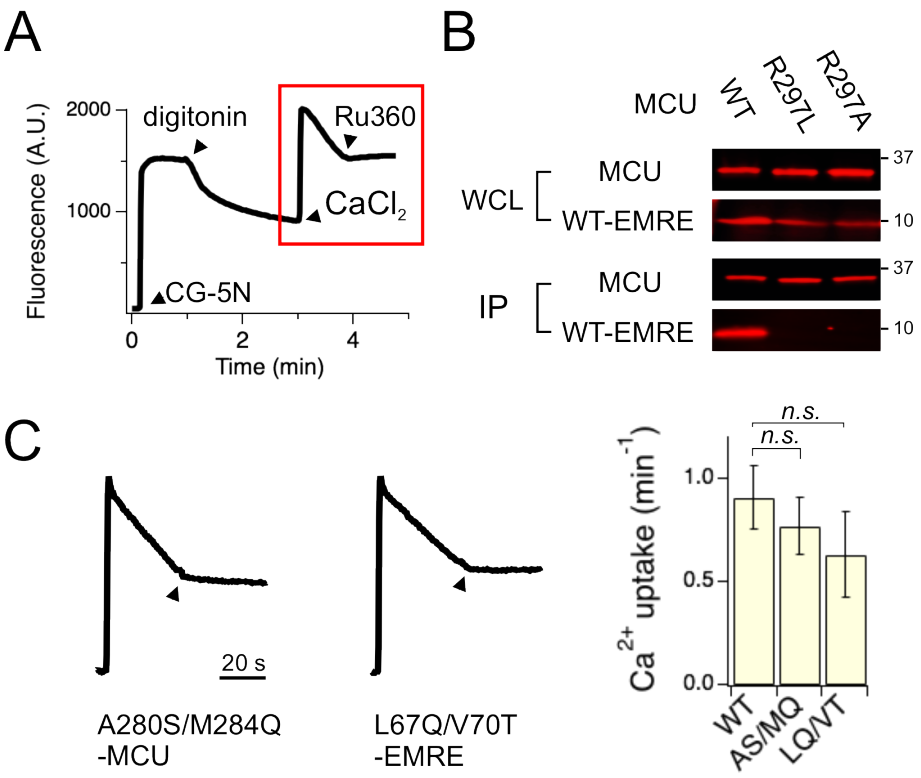

Figure S1. Roles of individual MCU-EMRE contact sites, Related to Figure 1. (A) A representative trace from a fluorophore-based Ca^{2+} flux experiment. The signal from calcium green 5N (CG-5N) in the cell suspension decreased to a steady state after digitonin permeabilization of cell plasma membranes. Adding 15-μM $CaCl_2$ then led to a sharp signal increase, followed by a signal decline that represents mitochondrial Ca^{2+} uptake, which can be inhibited by Ru360. Only the trace inside the red box was presented in other places in the manuscript. **(B)** Effect of R297 (site 1) mutations on the stability of the MCU-EMRE complex. Both R297A and R297L mutations drastically weaken EMRE binding to MCU. **(C)** Representative Ca^{2+} flux traces of site-2 mutants and a summary bar chart. A280S/M284Q-MCU was expressed in MCU-KO cells and L67Q/V70T-EMRE was expressed in EMRE-KO cells. Data are presented as mean ± SEM. *n.s.*, no significance (two-tailed t-test).

Figure S2

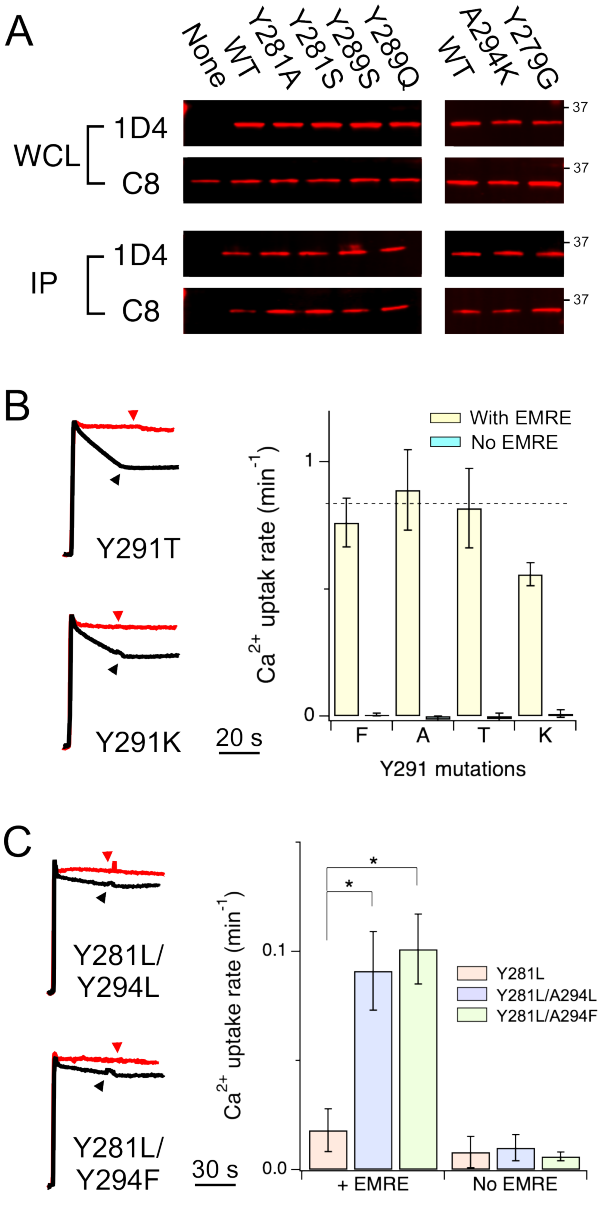

Figure S2. Biochemical and functional properties of MCU mutants, Related to Figure 4.
(A) CoIP experiment testing oligomerization of MCU mutants. 1D4-tagged WT or mutant MCU were used to pull down C8-tagged WT-MCU in ME-KO cells. *WCL*: whole cell lysate. *IP*: eluent. **(B)** Effects of Y291 mutations on Ca^{2+} transport. Mutants were expressed in MCU-KO cells (black) or ME-KO cells (red). Dashed line: mean Ca^{2+} uptake rate of WT hMCU. **(C)** Partial functional restoration of Y281L by Y294L or Y294F. Red: no EMRE. Black: with EMRE. Data are presented as mean ± SEM. * $p < 0.05$, as determined by two-tailed t-test.

Figure S3

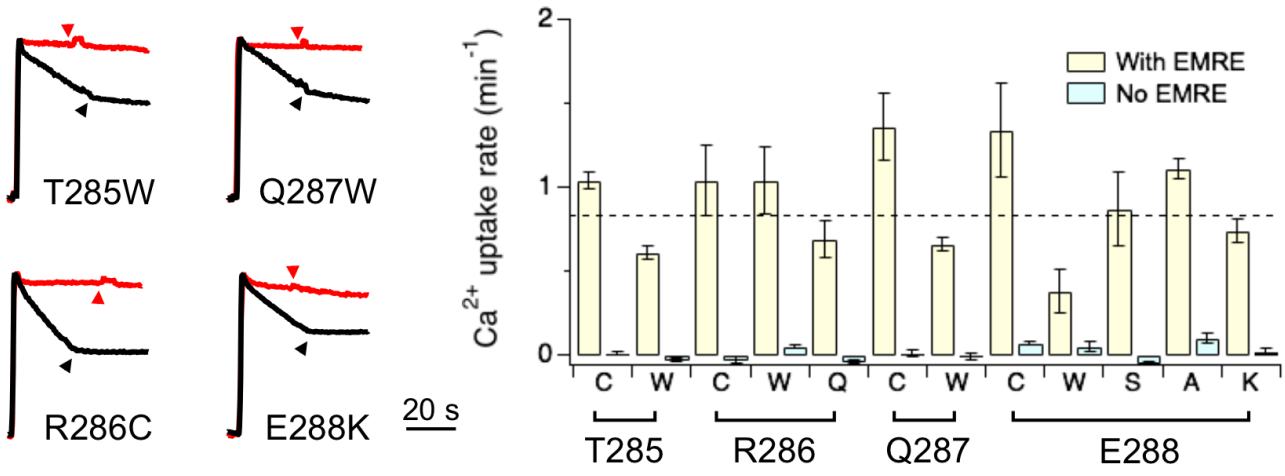

Figure S3. Functional importance of the JML, Related to Figure 4. Indicated MCU mutants were expressed in MCU-KO cells (black) or ME-KO cells (red). Dashed line: mean Ca^{2+} uptake rate of WT hMCU. Data are presented as mean ± SEM.

Figure S4

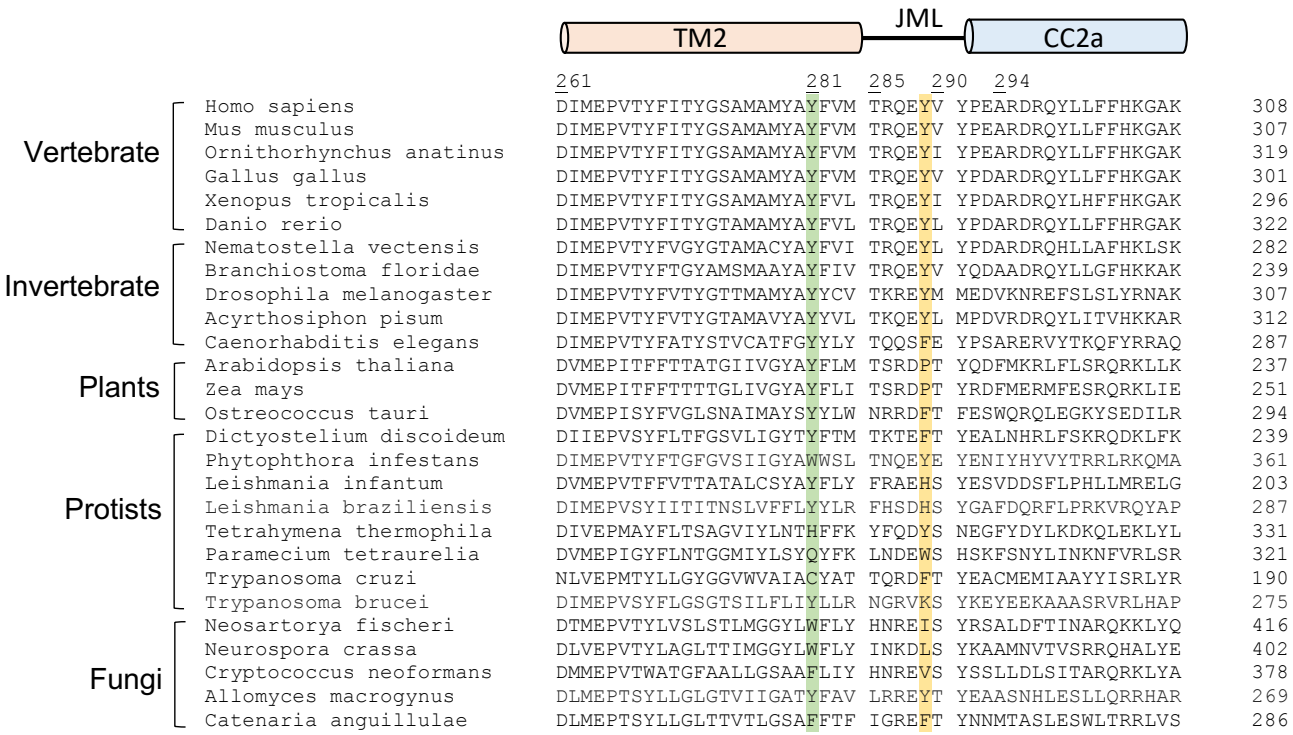

Figure S4. A sequence alignment of MCU homologues, Related to Figure 6. Numbers above the hMCU sequence indicate the position number of hMCU residues. Corresponding residues of Y281 and Y289 are highlighted. *A. macrogynus* and *C. anguillulae* belong to a small group of fungal species that contain EMRE.

Figure S5

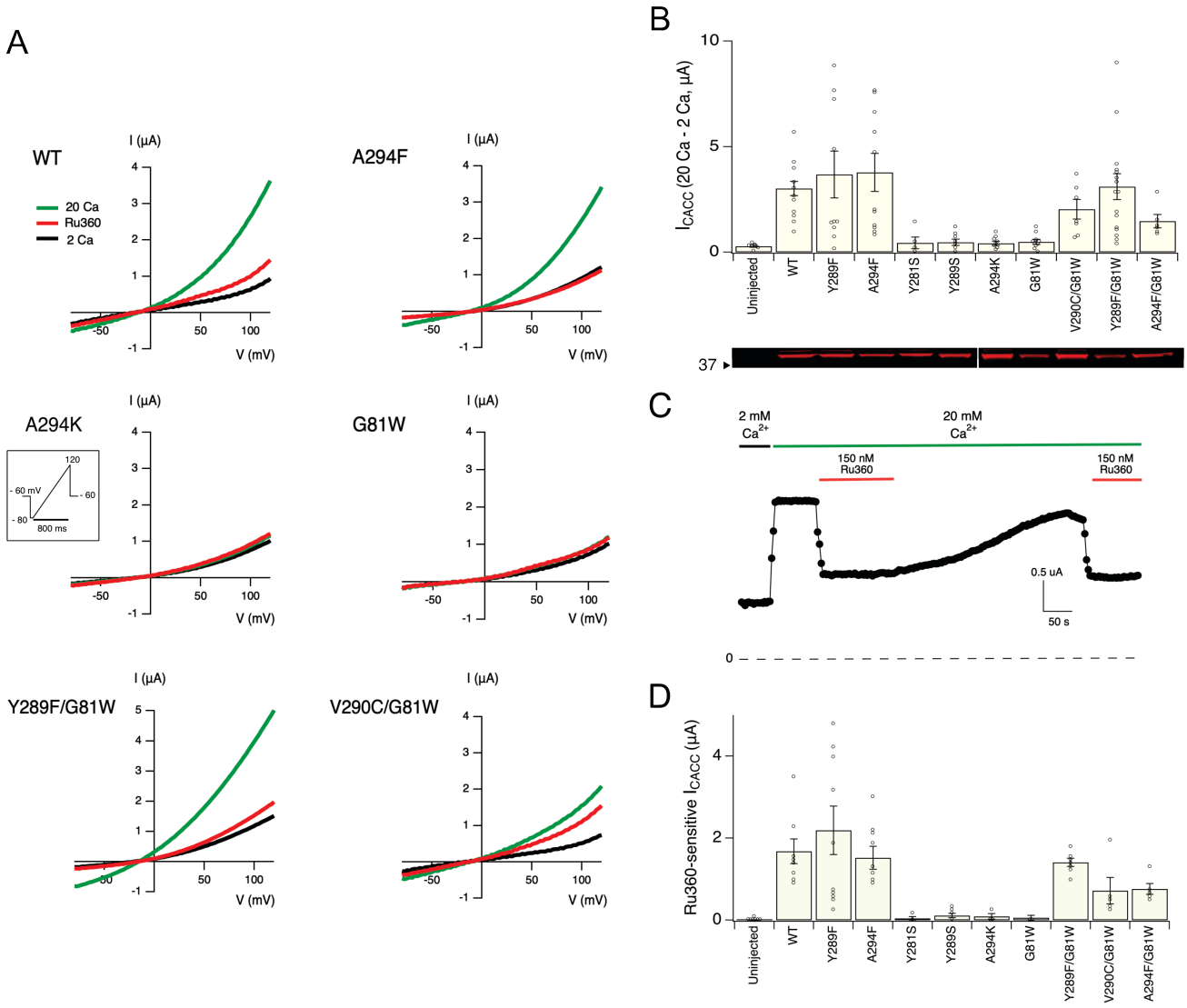

Figure S5. Two-electrode voltage clamp analysis of uniporter mutants, Related to Figure 7. (A) I-V relations of hME-induced I_{CACC}. Currents were obtained using repeated voltage ramps (inlet). In a typical experiment, oocytes were perfused with a 2-mM Ca^{2+} solution (ND96, black), followed by solution change to a 20-mM Ca^{2+} solution (Ca-20, green) and then to Ca-20 plus 150 nM Ru360 (red). **(B)** A bar chart summarizing the increase of I_{CACC} at 120 mV upon solution change from ND96 to Ca-20. The expression levels of tested mutants were shown in Western images below the bar chart. Mutants were labeled according to their corresponding mutations in MCU (or EMRE in the case of G81W). **(C)** The response of WT-hME-induced I_{CACC} to Ru360. I_{CACC} at 120 mV was plotted against time, showing that Ru360 rapidly and reversibly inhibits I_{CACC}. **(D)** A bar chart summarizing the amplitude of Ru360-inhibitable currents in Ca-20 at 120 mV. Data are presented as mean ± SEM.

Figure S6

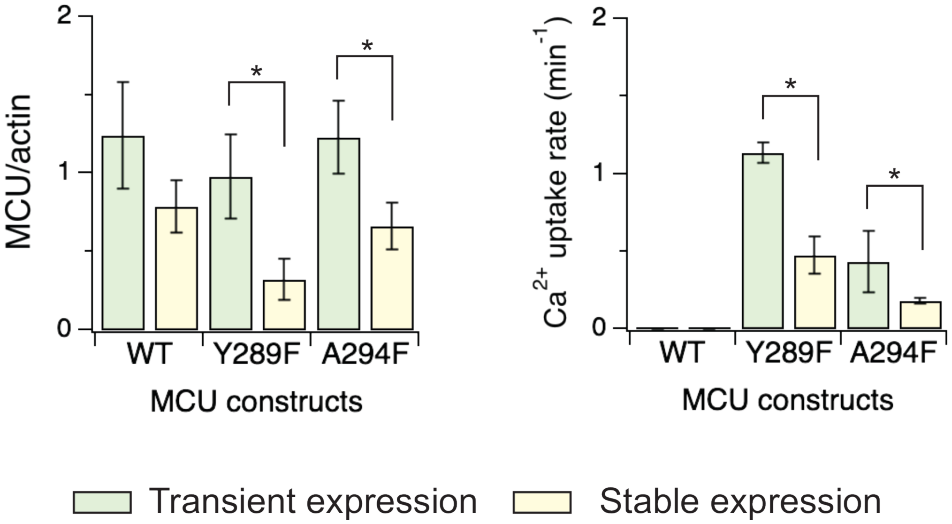

Figure S6. Comparison of MCU expression and Ca^{2+} flux in transient versus stable expression, Related to Figure 7. MCU constructs were expressed in ME-KO cells via transient or stable expression. Stable expression led to lower MCU expression (quantified as the MCU/actin Western signal ratio in the same membrane) and accordingly, slower rate of uniporter-mediated mitochondrial Ca^{2+} uptake. This helps explain why mitoplast patch-clamp recordings of stably expressed Y289F or A294F show a current density lower than that of WT-MCU transiently expressed in MCU-depleted cells (Fig. 7C-D). Moreover, the observation that uniporter-mediated mitochondrial Ca^{2+} uptake can be manipulated by MCU expression levels indicate that mitochondrial Ca^{2+} uptake is rate-limited by MCU expression. Data are presented as mean ± SEM. * $p < 0.05$ (two tailed t-test).

Figure S7

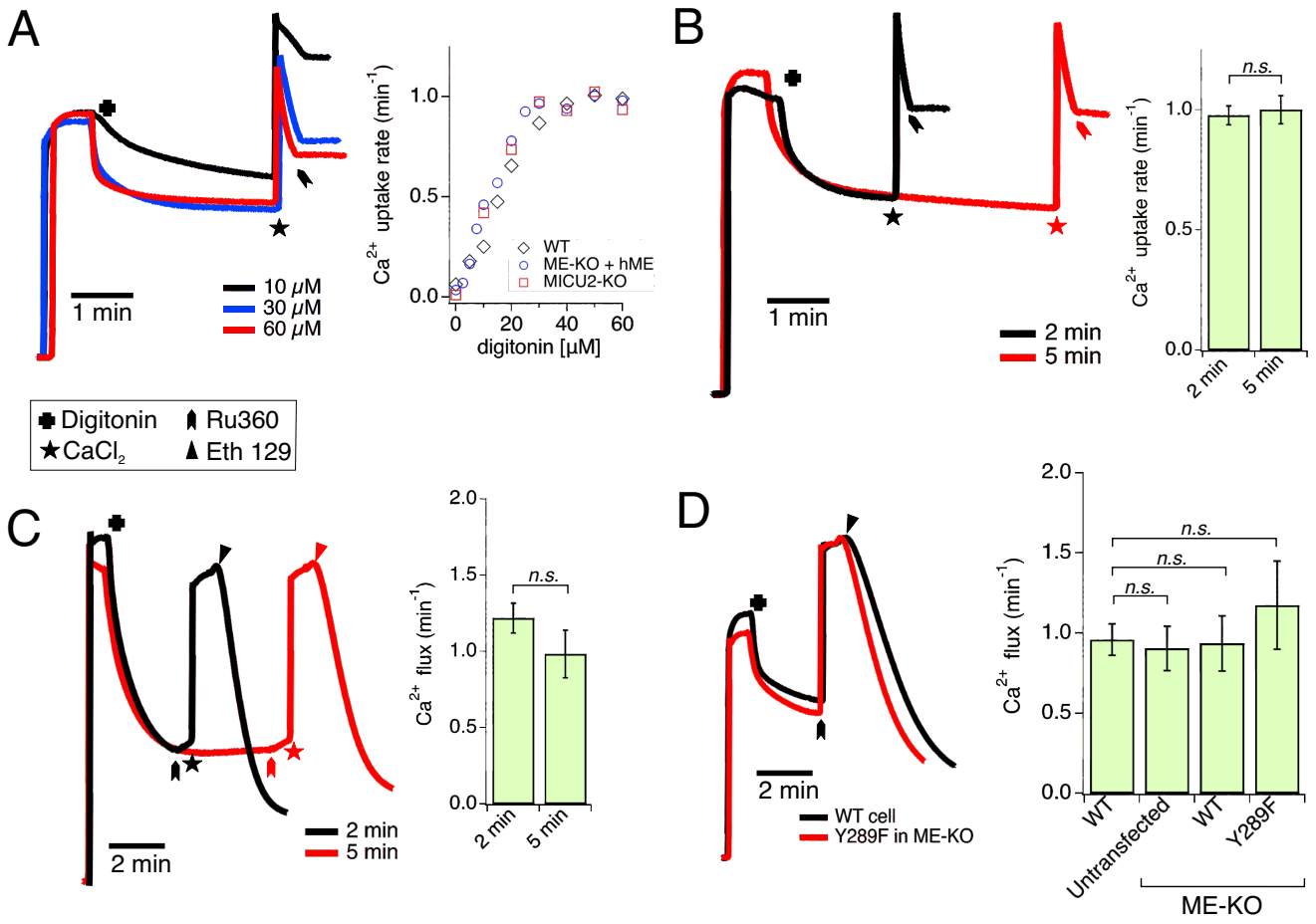

Figure S7. Additional control experiments for the Ca^{2+} flux assay, Related to STAR Methods. (A) Mitochondrial Ca^{2+} uptake measured using cells permeabilized with various concentrations of digitonin. The traces on the left were obtained with MICU2-KO cells. The scattered plot shows that WT, MICU2-KO cells, or ME-KO cells expressing a human MCU-EMRE fusion protein (hME) all have similar mitochondrial Ca^{2+} uptake in response to titration of digitonin, suggesting that digitonin permeabilizes these cells to a similar extent. **(B)** The effect of time lapse between adding digitonin and Ca^{2+} on mitochondrial Ca^{2+} uptake. The rates of Ca^{2+} uptake are identical with a 2- or 5-min time lapse, suggesting that a small variation regarding the timing of adding Ca^{2+} would not affect the result of the assay. **(C)** The effect of time lapse between adding digitonin and Ca^{2+} on the driving force for Ca^{2+} influx. Ru360 was added 2 min or 5 min after digitonin permeabilization, followed immediately by 10 μM Ca^{2+} and 2 μg/mL of ETH129, a Ca^{2+} ionophore. ETH129-mediated Ca^{2+} influx into mitochondria shows similar rates

with a 2- or 5-min time lapse after adding digitonin, suggesting that the driving force for Ca^{2+} influx remains identical 2 – 5 min after membrane permeabilization, and that digitonin permeabilization of the membranes reaches a steady state within 2 min. **(D)** ETH129-mediated Ca^{2+} influx into mitochondria under various conditions. Results show that the rates of Ca^{2+} influx are indistinguishable in WT or ME-KO cells, or ME-KO cells transiently expressing WT or Y289F MCU. Thus, deletion of MCU and EMRE, or transient expression does not affect the driving force for Ca^{2+} influx. Data are presented as mean ± SEM. *n.s.,* no significance (two-tailed t-test).