MICU1 Motifs Define Mitochondrial Calcium Uniporter Binding and Activity

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SUMMARY

Resting mitochondrial matrix Ca\(^{2+}\) is maintained through a mitochondrial calcium uptake 1 (MICU1)-established threshold inhibition of mitochondrial calcium uniporter (MCU) activity. It is not known how MICU1 interacts with MCU to establish this Ca\(^{2+}\) threshold for mitochondrial Ca\(^{2+}\) uptake and MCU activity. Here, we show that MICU1 localizes to the mitochondrial matrix side of the inner mitochondrial membrane and MICU1/MCU binding is determined by a MICU1 N-terminal polybasic domain and two interacting coiled-coil domains of MCU. Further investigation reveals that MICU1 forms homo-oligomers, and this oligomerization is independent of the polybasic region. However, the polybasic region confers MICU1 oligomeric binding to MCU and controls mitochondrial Ca\(^{2+}\) current (\(i_{\text{MCU}}\)). Moreover, MICU1 EF hands regulate MCU channel activity, but do not determine MCU binding. Loss of MICU1 promotes MCU activation leading to oxidative burden and a halt to cell migration. These studies establish a molecular mechanism for MICU1 control of MCU-mediated mitochondrial Ca\(^{2+}\) accumulation, and dysregulation of this mechanism probably enhances vascular dysfunction.

INTRODUCTION

Receptor-mediated cytosolic Ca\(^{2+}\) flooding is rapidly cleared by endoplasmic reticulum refilling, plasma membrane efflux, and mitochondrial Ca\(^{2+}\) uptake (Berridge et al., 2003; Soboloff et al., 2012). In most eukaryotic nonexcitable and excitable cells, carriers, exchangers, and channels regulate mitochondrial Ca\(^{2+}\) uptake during the resting and active states (Drago et al., 2011; Duchen et al., 2008; Nicholls, 2005; Rizzuto et al., 2012; Santodomingo and Dremaux, 2010; Williams et al., 2013). Following G-protein-coupled receptor or receptor-tyrosine-kinase-agonist stimulation, the mitochondrial Ca\(^{2+}\) uniporter (MCU) channel rapidly transports elevated cytosolic Ca\(^{2+}\) into the mitochondrial matrix utilizing the highly negative mitochondrial membrane potential (\(A\psi_m\)) (Bernardi, 1999; Gunter et al., 1994; Kirschok et al., 2004). Mitochondrial Ca\(^{2+}\) uptake stimulates bioenergetics through activation of Ca\(^{2+}\)-sensitive dehydrogenases and thus modulates ATP synthesis (Babcock et al., 1997; Balaban, 2009; Denton and McCormack, 1980; Duchen et al., 2008; Hajończyk et al., 1995; Hansford, 1994; McCormack et al., 1990). Reciprocally, mitochondrial Ca\(^{2+}\) overload promotes physical damage to mitochondria leading to bioenergetic crisis and cell death (Orenius et al., 2003; Szárai et al., 1999). Pathophysiological dysregulation of mitochondrial Ca\(^{2+}\) uptake has been linked to organ damage via chronic oxidative burden, autophagy, and cellular dysfunction (Baines et al., 2005; Cárdenas et al., 2010; Clapham, 2007; Joiner et al., 2012; Lemasters et al., 2009; Mallilankaraman et al., 2012b).

Mitochondrial Ca\(^{2+}\) uptake has been functionally described for decades, but only recently using integrative bioinformatics and RNAi screening approaches, three components of the MCU complex (mitochondrial calcium uptake 1 [MICU1], MCU, and MCU regulator 1) were identified that together constitute the activity of MCU (Baughman et al., 2011; De Stefani et al., 2011; Mallilankaraman et al., 2012a, b; Perocchi et al., 2010). Although the mitochondrial uniporter complex tightly regulates Ca\(^{2+}\) influx, the molecular mechanisms of uniporter regulation are unclear. This unknown regulatory mechanism stems from the observation that mitochondrial Ca\(^{2+}\) influx exhibits a finite trigger proposed as a “set point” in 1978 (Nicholls, 1978, 2005). The set point is overcome and the mitochondria begins Ca\(^{2+}\) uptake at cytosolic Ca\(^{2+}\) levels exceeding ~3 \(\mu\)M. The trigger was characterized as the function
Figure 1. Protein Flux Dynamics Illustrate MICU1 Is Localized in the Mitochondrial Matrix Compartment
(A) HeLa cells were cotransfected with cytochrome c-GFP and COX8a-mRFP (MTS) plasmid constructs.
(B) Similarly, HeLa cells were cotransfected with MICU1-YFP and COX8-mRFP (MTS).
(C and D) Plasma membranes of HeLa cells were briefly permeabilized with digitonin (0.002% w/v) containing intracellular medium for 7 min. Cells were treated with mastoparan (20 μg/ml) to trigger outer mitochondrial membrane permeabilization. Protein flux dynamics were visualized using confocal microscopy.

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of MCU-associated protein MICU1 which is able to suppress mitochondrial Ca$_{2+}$ uptake up to the cytosolic levels ~3 μM Ca$_{2+}$ (Malilankaraman et al., 2012b). Thus far, MICU1 is the only known protein that interacts with MCU to prevent mitochondrial Ca$_{2+}$ uptake under resting conditions (Malilankaraman et al., 2012b), but the MICU1 localization and the binding domains of the MICU1/MCU complex that establish the set point are unknown.

Here, we show that MICU1 interacts with MCU in the mitochondrial matrix and MICU1/MCU binding is determined by a MICU1 N-terminal polybasic domain and two coiled-coil MCU domains. MICU1 EF hands regulate MCU channel activity, but not MICU1/MCU binding. Additionally, loss of MICU1 induces MCU activity leading to vascular endothelial oxidative stress and diminished endothelial cell migration.

### RESULTS

**Protein Flux Analysis Establishes MICU1 Is Compartmentalized in the Mitochondrial Matrix**

The MCU pore subunit is located in the inner mitochondrial membrane (IMM), and thus its regulator, MICU1, must be present either in the intermembrane space or the matrix side of the mitochondrial inner membrane. To determine the suborganelar localization of MICU1, we developed a dynamic protein flux assay using confocal microscopy in which the outer mitochondrial membrane (OMM) or both the OMM and IMM of the mitochondria were selectively permeabilized and release of MICU1 was subsequently visualized from the mitochondrial compartments. HeLa cells were cotransfected with expression vector pairs encoding yellow fluorescent protein (YFP)-tagged MICU1 and monomeric red fluorescent protein (mRFP)-tagged mitochondrial targeting sequence of matrix-localized cytochrome c oxidase subunit 8 (COX8) (Figure 1B) or mitochondrial intermembrane space protein, GFP-tagged cytochrome c (cyto c) cotransfected with mRFP-COX8 (Figure 1A). Combinations of both cyto c/COX8 and MICU1/COX8 showed general mitochondrial localization (Figures 1A and 1B). To visualize the MICU1 compartmentalization, the plasma membrane was permeabilized using 0.002% digitonin (20 μg/ml) and permeabilized cells were bathed in digitonin-free intracellular medium (ICM) before exposure to mitochondrial permeabilizing agents.

The cyto c/COX8 cotransfected permeabilized cells were subjected to mastoparan (20 μg/ml), a wasp venom peptide toxin that induces OMM permeabilization (Kluck et al., 1999; Pfeiffer et al., 1995). The release of intermembrane-space-resident proteins due to mastoparan was visualized by confocal microscopy in real time. OMM permeabilization by mastoparan rapidly released cyto c, but not matrix-localized COX8 (Figures 1C and 1E). Similarly, MICU1-YFP/COX8-mRFP permeabilized cells were exposed to mastoparan. We found that mitochondrial localized MICU1 and COX8 were not released from the mitochondrial intermembrane space (Figures 1D and 1F). We next asked whether permeabilization of both the OMM and the IMM promotes MICU1 release from the mitochondrial matrix. Permeabilized cells were exposed to a fungal peptide, alamethicin (20 μg/ml), that induces large pores in both mitochondrial membranes (Kluck et al., 1999). Permeabilization of both membranes induced the release of COX8 from the matrix in addition to cyto c from the intermembrane space (Figures 1G, 1I, and 1P). It has been recently reported that MICU1 exists in the intermembrane space (Csordás et al., 2013), but similar to COX8, MICU1 was released only upon permeabilization of both the OMM and IMM by alamethicin (Figures 1H, 1J, and 1P). The integral inner membrane channel pore subunit MCU was not released by mastoparan or alamethicin permeabilization (Figures 1K–1P). The dynamic protein flux assay was complemented by western blot analysis. As expected, cyto c was released by both mastoparan and alamethicin and appeared in the cytosolic supernatant (Figure 1Q). MICU1 was only released by alamethicin, whereas integral membrane MCU was not released by either mastoparan or alamethicin (Figure 1Q). A soluble mitochondrial matrix protein heat shock protein 60 (HSP60) was used as a substitute for COX8 (Figure 1Q). Further, we examined whether MCU and MICU1 diffuse rapidly after photobleaching. Photobleaching of MICU1, but not MCU, showed rapid fluorescence recovery after photobleaching (FRAP). This result suggested that MICU1 is less associated with the IMM than MCU (Figure 1R). These results reveal that MICU1 is compartmentalized in the mitochondrial matrix side of the IMM.

**Mapping of MICU1- and MCU-Binding Regions Reveals Conserved MICU1 Polybasic Motif Domains, but Not EF Hand Domains, Are Essential for MCU Binding**

Although MICU1 and MCU form a complex (Malilankaraman et al., 2012b; Perocchi et al., 2010), it is not known which regions of MICU1 and MCU determine binding. To map the binding
regions of MICU1 and MCU, we generated five hemagglutinin (HA)-tagged MICU1 truncation mutants (Figure 2A) and transfected these into COS-7 cells stably expressing GFP-tagged full-length MCU. The transfected cell lysates were subjected to immunoprecipitation and western blot analysis. Immunoprecipitation of GFP-tagged MCU pulled down all MICU1 truncation mutants except deletion of amino acids 131–200 (MICU1-Δ2) (Figure 2B). Intriguingly, the MICU1-Δ1 (deletion of amino acids 61–130) partially interacts with wild-type MCU (Figure 2B). Although the effect of the MICU1-Δ2 deletion was profound, lack of any interaction suggests a conformational deformity of MICU1-Δ2 as a result of the truncation. In contrast, the partial interaction of MICU1-Δ1 suggests that the protein is maintaining an interacting tertiary structure that is recognized by MCU. Therefore, we examined the MICU1-Δ1 amino acid sequence for evolutionarily conserved interaction motifs and found a series of positively charged lysine residues (amino acids [aa] 99–110) (Figures S1A and S1B) which corresponds to the consensus sequence of a polybasic region motif which is known as not only an interaction motif but also as a membrane-anchoring motif (Hancock et al., 1990; Heo et al., 2006; Papayannopoulos et al., 2005; Williams, 2003). The polybasic N-terminal region domain was mutated to polyglutamate (MICU1-ΔK) (Figure 2C), and MICU1-ΔK reduced MCU interactions (Figure 2D); however, MICU1-ΔK still localized to the mitochondria (Figures S1D and S1E). The marked, but not complete, loss of interaction with MCU suggests the MICU1 polybasic region motif is a determinant of MICU1/MCU interaction. The profound loss of binding in MICU1-Δ2 may be a consequence of the deleted region’s proximity to an EF hand of MICU1. To determine if the EF hands are necessary for MCU binding, both EF hands were mutated (Figure 2C). Although functionally important (Malilihankaran et al., 2012b; Perocchi et al., 2010), the EF hands of MICU1 did not determine MCU binding (Figure 2D). This result also demonstrates that although MICU1-ΔK has functional EF hands, it failed to interact with MCU (Figure 2D). We next asked whether EF hands’ Ca$^{2+}$ binding domains may participate in MCU/MICU1 binding. We therefore tested whether Ca$^{2+}$ determines MICU1/MCU binding using MICU1 full-length and single and double EF1 and EF2 mutants. Our coimmunoprecipitation data demonstrated that MICU1/MCU binding is independent of [Ca$^{2+}$] (Figure 2E). Together, these results indicate that the polybasic region, but not the EF hands of MICU1, are determinant of MCU binding.

**MCU Coiled-Coil Domains Are Determinants of MICU1 Binding**

Next to map which regions of MCU bind MICU1, we created three MCU truncation mutants stably expressing in COS-7 cells that were lacking aa 150–220 (MCU-Δ1), aa 291–320 (MCU-Δ2), or aa 321–351 (MCU-Δ3; Figure 3A). Then, these stable cells were transfected with full-length MICU1-HA plasmid constructs. The pull-down of full-length MICU1-HA was unable to immunoprecipitate MCU-Δ1 and MCU-Δ3 suggesting that these regions corresponding to MCU coiled-coil domains are essential for MICU1 binding (Figures 3B and S1C). To validate the coimmunoprecipitation data, we performed fluorescence resonance energy transfer (FRET) acceptor photobleaching to determine whether MCU-Δ1 and Δ3 interact with MICU1. Coexpression of mitochondrial marker COX8A-mRFP and MCU-GFP constructs revealed that MCU-Δ1, Δ2, and Δ3 mutants did not alter the mitochondrial targeting (Figure S2). Comparable protein expressions of wild-type, Δ1, Δ2, and Δ3 MCU-GFP were selected for FRET analysis. Expectedly, MCU-GFP and MICU1-YFP interact; however, neither MCU-GFP nor MICU1-YFP interact with matrix-localized COX8A-RFP (Figures 3C–3E). Similar to coimmunoprecipitation analysis, MCU wild-type and MCU-Δ2, but not MCU-Δ1 and MCU-Δ3, interact with MICU1 (Figures 3C and 3F–3H). Further, we have also conducted interaction studies by yeast two-hybrid assay (Figure 3I). In support of coimmunoprecipitation and FRET, MCU wild-type and MCU-Δ2 showed positive blue colonies, but MCU-Δ1 and MCU-Δ3 did not, indicating that MCU wild-type and MCU-Δ2 interact with MICU1 (Figure 3J). Collectively, these results demonstrate that MCU-Δ1 and MCU-Δ3, regions corresponding to the coiled-coil domains, are necessary for interaction with MICU1.

**MICU1 Polybasic Region and EF Hands Regulate MCU-Mediated Mitochondrial Ca$^{2+}$ Uptake**

We next sought to characterize whether MICU1 undergoes oligomerization as a mechanistic step in regulation of MCU-mediated Ca$^{2+}$ uptake. To evaluate the possibility of MICU1 oligomerization, we developed a coimmunoprecipitation strategy using cotransfection of two MICU1 constructs (MICU1-HA and MCU1-Flag) in COS-7 cells. Coimmunoprecipitation of MICU1-Flag with HA antibody indicated that MICU1-HA and MICU1-Flag are able to form oligomeric complexes (Figure 4A, right panel). Similarly, COS-7 cells were cotransfected with MICU1-Flag and MCU1-ΔK-YFP plasmid constructs. Coimmunoprecipitation of MICU1-Flag with GFP antibody indicated that MICU1-Flag is still able to oligomerize with MICU1-ΔK (Figure 4B, right panel). These results demonstrate that the polybasic region of MICU1 is not a MICU1/MICU1 interaction domain but instead supports the polybasic region’s role in MCU interaction.

Having determined the binding regions of MCU and MICU1 through a series of deletion and mutation analysis, we next asked whether MICU1-ΔK and MICU1-ΔEF1ΔEF2 affect MCU-mediated [Ca$^{2+}$]$_{m}$ uptake. Permeabilized control, MCU1-ΔK, and MICU1-ΔEF1ΔEF2-expressing HeLa cells were loaded with Fura-FF to determine [Ca$^{2+}$]$_{m}$ uptake rate as a decay of extramitochondrial Ca$^{2+}$ and JC-1 to simultaneously monitor mitochondrial membrane potential ($\Delta \Psi_m$) dynamics. MICU1 actively inhibits MCU at cytosolic Ca$^{2+}$ levels < 3 μM (Malilihankaran et al., 2012b). Therefore, to determine the phenotype of the MICU1-ΔK, HeLa cells were challenged with a 1 μM Ca$^{2+}$ bolus, well within the functional inhibitory range of wild-type MICU1. We found that MICU1-ΔK cells rapidly cleared the 1 μM Ca$^{2+}$ bolus under normal $\Delta \Psi_m$ indicating the loss of MICU1 inhibition of MCU activity at <3 μM. Conversely, control cells did not exhibit MCU-dependent Ca$^{2+}$ uptake (Figures 4C–4F), because the MICU1 inhibition was intact. Next, we examined the total amount of Ca$^{2+}$ buffered by control and MICU1-ΔK mitochondria using the mitochondrial uncoupler, CCCP. MICU1-ΔK-overexpressing cells accumulated large amounts of Ca$^{2+}$ (Figure 4G) suggesting that MICU1-ΔK is unable to properly interact with MCU to establish the set point for MCU-mediated Ca$^{2+}$ uptake. Functionally,
Figure 2. Mapping the MICU1 Interaction Domains with MCU

(A) Scheme depicts full-length MICU1 and truncation constructs.

(B) Stably MCU-GFP-expressing COS-7 cells were transfected with HA-tagged full-length MICU1 or truncations. Following immunoprecipitation with GFP antibody, total cell lysates and immunoprecipitated materials were subjected to western blot analysis. Cell lysates were probed with HA (top left) or GFP (bottom left) antibodies to serve as inputs. Immunoprecipitated samples were probed with HA (top right) or GFP (bottom right) antibodies. GFP antibodies coimmunoprecipitate full-length MICU1, D3, D4, and D5 MICU1-HA truncations, but not MICU1-D1 and MICU1-D2. n = 3–5. IP, immunoprecipitation; WB, western blot.

(C) Scheme depicts full-length MICU1 and MICU1-D1EF1, MICU1-D1EF2, MICU1-D1EF1D2, and MICU1-DK mutant constructs. WT, wild-type.

(D) Stably MCU-GFP-expressing COS-7 cells were transfected with Flag-tagged full-length MICU1 and mutants. Following immunoprecipitation with GFP antibody, total cell lysates and immunoprecipitated materials were subjected to western blot analysis. Cell lysates were probed with Flag (top left) or GFP (bottom left) antibodies to serve as inputs. Immunoprecipitated samples were probed with Flag (top right) or GFP (bottom right) antibodies. GFP antibodies coimmunoprecipitate full-length MICU1, MICU1-D1EF1, MICU1-D1EF2, and MICU1-D1EF1D2, but not MICU1-DK mutant. n = 3.

(E) As described in (D), stably MCU-GFP-expressing COS-7 cells were transfected with Flag-tagged MICU1-D1EF1, MICU1-D1EF2, and MICU1-D1EF1D2 mutants. Cell lysates were incubated with or without 50 μM Ca²⁺, immunoprecipitation was performed with GFP antibody, and cell lysates were subjected to western blot analysis. Cell lysates were probed with Flag (top left) or GFP (bottom left) antibodies to serve as inputs. Immunoprecipitated samples were probed with Flag (top middle and right) or GFP antibodies (bottom middle and right).
MICU1 EF hands are the Ca\textsuperscript{2+} sensor of the MCU complex, and although EF hand mutants bind MCU (Figure 2D), the mutations of EF1, EF2, or EF1/EF2 (Figure 2C) led to dysfunction with a functional phenotype similar to MICU1 knockdown (KD) (Mallankaraman et al., 2012b) in which the mitochondria constitutively accumulate Ca\textsuperscript{2+} that would otherwise remain as cytosolic Ca\textsuperscript{2+} (Figures 4H–4L). These data indicate that both polybasic and EF hands are essential for MICU1 regulation of MCU.
Because MCU-mediated mitochondrial Ca\(^{2+}\) uptake is regulated by MICU1, we examined whether MICU1-DK or MICU1-DE1DE2 mutants were able to regulate MCU current (I\(_{\text{MCU}}\)) in HeLa cells. To measure I\(_{\text{MCU}}\), we utilized mitoplast patch-clamp (Chaudhuri et al., 2013; Fieni et al., 2012; Joiner et al., 2012; Kirichok et al., 2004). As a first step, freshly isolated mitochondria were loaded with ΔΨ\(_{\text{m}}\) indicator, rhodamine 123, and ratiometric DJ\(_{\text{m}}\) indicator Fura-2FF. 

**Figure 4. MICU1 Binding and Functional Domains Regulate MCU-Mediated Ca\(^{2+}\) Uptake**

(A) COS-7 wild-type cells were cotransfected with HA-tagged full-length MICU1 and Flag-tagged full-length MICU1 plasmid constructs. Following immunoprecipitation with HA antibody, total cell lysates and immunoprecipitated materials were subjected to western blot analysis. Cell lysates were probed with HA (left) or Flag antibodies (right panel, first three lanes) to serve as inputs. Immunoprecipitated samples were probed with Flag (right panel, last three lanes). n = 3.

(B) COS-7 wild-type cells were cotransfected with Flag-tagged full-length MICU1 and YFP-tagged MICU1-DK plasmid constructs. Following immunoprecipitation with GFP antibody, total cell lysates and immunoprecipitated materials were subjected to western blot analysis. Cell lysates were probed with GFP (left) or Flag (right panel, first three lanes) antibodies to serve as inputs. Immunoprecipitated samples were probed with anti-Flag (right panel, last three lanes). n = 3.

(C and D) HeLa cells were transfected with MICU1-DK plasmid, and stable cells were generated after G418 selection. MICU1-DK and wild-type cells were permeabilized with 0.004% digitonin in ICM buffer and loaded with extramitochondrial ratiometric Ca\(^{2+}\) indicator Fura-2FF. These cells were also loaded with ratiometric ΔΨ\(_{\text{m}}\) indicator JC-1. Cells were then challenged with 1 μM Ca\(^{2+}\) bolus. Finally, total mitochondrial Ca\(^{2+}\) uptake and ΔΨ\(_{\text{m}}\) dissipation were determined by uncoupler CCCP. Mean ± SEM; n = 3–6.

(E) Quantitation of wild-type and MICU1-DK extramitochondrial [Ca\(^{2+}\)]\(_{\text{out}}\) (to 450 s). Mean ± SEM; **p < 0.01; n = 3–6.

(F) Quantitation of 1 μM Ca\(^{2+}\) bolus uptake, [Ca\(^{2+}\)]\(_{\text{m}}\). Mean ± SEM; ***p < 0.001, n = 3–6.

(G) Quantitation of mitochondrial Ca\(^{2+}\) release following CCCP. Mean ± SEM; **p < 0.01, n = 3–6.

(H–L) Similar to MICU1-DK, EF hand double-mutant ΔEF1ΔEF2 was assessed for MCU-mediated Ca\(^{2+}\) uptake and ΔΨ\(_{\text{m}}\) dynamics. Mean ± SEM; **p < 0.01, ***p < 0.001, n = 3–6.
the mitoplast preparation was confirmed by confocal imaging (Figure 5A, inset). In the patch-clamp of whole-mitoplast configuration, the addition of 2 mM Ca\(^{2+}\) to the bath elicited an inwardly rectifying Ca\(^{2+}\) current that is almost completely blocked by 50 mM Ru360 (Kirichok et al., 2004; Figure 5A). Next, we examined if knockdown of human MICU1 or MCU proteins altered \(i_{\text{MCU}}\). Compared to control conditions, \(i_{\text{MCU}}\) in mitoplasts from MCU knockdown (MCU KD) cells was significantly lower (Figures 5B and 5D) which is correlated with MCU-mediated Ca\(^{2+}\) uptake (Figures S3A–S3C). In sharp contrast, stable knockdown of MICU1 exhibited larger \(i_{\text{MCU}}\) (Figures 5B and 5D). Finally, we conducted mitoplast patch-clamp recordings in MICU1-ΔK and MICU1-ΔEF1ΔEF2 mutants overexpressing HeLa cells. The electrophysiology revealed that MICU1-ΔK and ΔEF1ΔEF2 mitoplasts exhibited larger MCU current densities when compared to MCU currents from wild-type (Figures 5C and 5D). Having demonstrated the MCU Δ1 and Δ3 role in MICU1 binding, we next tested whether deletion of coiled-coil domains alter \(i_{\text{MCU}}\). MCU number 861 small hairpin RNA (shRNA)-insensitive MCU full-length and mutant constructs were stably expressed in MCU KD HeLa cells, and \(i_{\text{MCU}}\) was measured (Figures S3D–S3F). Reconstitution of full-length MCU in MCU KD mitoplasts re-established \(i_{\text{MCU}}\) (Figures S3E and S3F). Interestingly, MCU-Δ1 and MCU-Δ3 generated larger \(i_{\text{MCU}}\) than MCU-Δ2 indicating that MCU-Δ1 and -Δ3 regions are necessary for MICU1-mediated regulation of MCU activity. Taken together, these data strongly support that loss of MICU1 polybasic or EF hands increase the \(i_{\text{MCU}}\) exhibiting dominant negative phenotypes.

Loss of MICU1 Promotes Mitochondrial Ca\(^{2+}\) Accumulation and Oxidative Burden, Halting Endothelial Cell Migration

The ability of MICU1 to suppress MCU-mediated basal mitochondrial Ca\(^{2+}\) accumulation suggests that MICU1, rather than the MCU pore, is sensing Ca\(^{2+}\) uptake from the cytosol (Mallilankaraman et al., 2012b). Ablation of MICU1 causes mitochondrial Ca\(^{2+}\)-dependent chronic oxidant elevation and cellular stress. The MICU1 and MCU expression profiles in cell lines and primary cells indicated that human aortic endothelial cells have relatively high levels of MICU1 mRNA when compared to other cell types (Figures S4A and S4B; Aichberger et al., 2005). Nevertheless, MCU mRNA levels were relatively unchanged (Figures S4A and S4B). Therefore, we tested whether MICU1 downregulation impacts pathophysiological endothelial cell (EC) function. Because EC function is compromised in various cardiovascular disease states (Libby et al., 2006), we performed quantitative RT-PCR (qRT-PCR) to assess MICU1 expression in human control and cardiovascular disease (CVD)-derived primary ECs (Table S1). MICU1, but not MCU, mRNA levels were markedly downregulated in CVD ECs (Figure 6A). Basal \([\text{Ca}^{2+}]_{\text{cyt}}\) was considerably elevated in ECs derived from CVD patients when compared to control ECs. Further, reconstitution of MICU1 in CVD ECs reduced the \([\text{Ca}^{2+}]_{\text{cyt}}\) accumulation (Figures 6B and 6C). These results were further confirmed by the reduction of MICU1 protein in patients (Figure 6D). To examine if loss of MICU1 led to more MCU activity, mitochondria were isolated and mitoplast patch-clamp was performed. CVD ECs exhibited...
higher current density (Figures 6E and 6F) suggesting that the increased Ca\(^{2+}\) uptake is a result of increased open probability of MCU due to lower expression of MICU1 (Figures 6A and 6D). Aberrant basal mitochondrial Ca\(^{2+}\) uptake may lead to mitochondrial oxidative stress in the form of superoxide, which is produced when electrons leak from the electron transport chain to react with molecular oxygen. Ablation of MICU1 in the EC cell line, EA.hy926, increased mitochondrial superoxide levels (Figures S4C–S4E). In CVD ECs, loss of MICU1 resulted in higher [Ca\(^{2+}\)]\(_{\text{m}}\), through altered MCU-mediated mitochondrial Ca\(^{2+}\) uptake which subsequently increased basal superoxide levels (Figures 6G and 6H). We next tested whether elevated reactive oxygen species (ROS) from MICU1 knockdown lowers anti-oxidant levels and alters EC proliferation (Balaban et al., 2005). Total reduced glutathione (GSH) content was assessed in EA.hy926 ECs expressing MICU1 shRNA or MICU1 shRNA + shRNA-insensitive MICU1. Reconstitution of MICU1 in MICU1 KD cells prevented the chronic ROS-induced reduction of GSH levels (Figures S4F and S4G). Further, reduced cell proliferation in MICU1-silenced ECs was restored by reconstitution with shRNA-insensitive MICU1cDNA (Figures S4H and S4I). Another key functional question is whether loss of MICU1 leads to altered angiogenesis in CVD. CVD-derived ECs exhibited diminished migration, which was rescued by stable expression of MICU1 (Figures 6I and 6J). We next tested whether CVD-derived ECs have an altered monocyte adhesion phenotype.
We observed that CVD-derived ECs have an inherent propensity for monocyte adhesion (Figures S4J and S4K).

To explore the human MICU1 phenotype, we silenced MICU1 in mice and investigated the mitochondrial Ca\(^{2+}\) handling and vascular integrity. In vivo lentiviral delivery of MICU1 shRNA effectively knocked down MICU1 mRNA levels in ECs (Figure 7A). We also tested the MICU1 mRNA levels in cardiomyocytes, smooth muscle cells (SMCs), and splenocytes. Although SMCs and splenocytes, but not cardiomyocytes, showed partial knockdown of MICU1 following shRNA delivery, the MICU1 knockdown is more pronounced in ECs (Figure S5). Similar to cell-based and CVD-derived studies, downregulation of MICU1 expression promoted higher basal \([Ca^{2+}]_m\) accumulation and ROS production (Figure 7B), culminating as a loss of vascular integrity (Figures 7C and 7D). Collectively, these results show that loss of MICU1 leads to aberrant EC mitochondrial Ca\(^{2+}\) handling and vascular pathology.

**DISCUSSION**

These findings establish that mitochondrial matrix resident, MICU1, forms regulatory oligomers that require the MICU1 polybasic region for MCU binding and suppression of \(I_{\text{MRCU}}\). However, the polybasic region of MICU1 does not determine MICU1 oligomerization. Additionally, MICU1 EF hands which confer functional regulation of \(I_{\text{MRCU}}\) do not determine MICU1/MCU complex formation. Our experiments also establish that the coiled-coil domains of MCU are essential for MICU1 binding.

Identifying the physiological function of protein regions remains a challenge for advanced bioinformatics and requires broad-based biochemical techniques. One such technique, the protein dynamic flux assay, provided a tool to discover MICU1 protein localization in the mitochondria while circumventing current conventional microscopy resolution limits. The major advantages of this method are that it is rapid, specific, real-time imaging, without RNAi silencing, subcellular fractionation, or antibodies, and it can resolve compartmentalization-determined protein functions such as residues of MCU accessible to MICU1. Taking the next step from localization, we show that the N-terminal region of the MICU1 polybasic motif determines MICU1/MCU binding. Whereas the polybasic motif has been previously reported to enhance protein-protein interaction (Williams, 2003), the polybasic motif is now identified in the mitochondria. The dominant negative MICU1-ΔK oligomer phenotype is likely a result of MICU1-ΔK interacting with wild-type MICU1, either sequestering wild-type MICU1 or causing a reduction of MICU1 oligomeric complex-binding affinity for MCU. Together, these data suggest that the polybasic region of MICU1 is not a MICU1/MICU1 interaction domain but supports the polybasic region’s role in MCU interaction. The loss of MICU1 regulation by polybasic mutation could also be explained by diminished interaction of the polybasic mutant with the inner leaflet of the IMM. In support of this, the conserved polybasic region contains a glycine residue that could undergo myristoylation for membrane anchoring (Mumby et al., 1990; Thelen et al., 1991). This anchor could provide a mechanism for MICU1 to come on and off at active and resting state. This loose association is supported by our FRAP studies which show that MICU1 as a population is more mobile than MCU (Figure 1R). These critically important and diverse roles of the polybasic region and its role in mitochondrial Ca\(^{2+}\) signaling will certainly be elucidated in future studies.

Despite physiological mitochondrial Ca\(^{2+}\) uptake participates in mitochondrial bioenergetics and cellular Ca\(^{2+}\) signaling,
chronic Ca\(^{2+}\) accumulation is known to elicit mitochondrial dysfunction through mitochondrial permeability transition pore opening, mitochondrial ROS elevation, and cell death (Duchêne et al., 2008; Hajnóczky et al., 2009). The mitochondrial Ca\(^{2+}\)-dependent cellular damage pathways are shared in many pathophysiological models, like cardiovascular disease, aging, and neurodegeneration (Davidson and Duchêne, 2007). In cardiovascular diseases, the endothelium suffers from Ca\(^{2+}\)-dependent damage (Davidson and Duchêne, 2007). Our results establish that endothelial cells derived from CVD subjects show low levels of MICU1 (Figures 6 and 7) and that the mitochondrial Ca\(^{2+}\)-uptake component of the disease phenotype can be reversed by the reconstitution of MICU1, thus restoring endothelial cell migration.

Collectively, we established a unique resolution-independent confocal technique to characterize the localization of complex components in viable organelles. Most importantly, we revealed that MICU1 interacts with MCU and regulates MCU-dependent [Ca\(^{2+}\)]\(_{\text{m}}\) uptake with a polybasic region and Ca\(^{2+}\)-sensing EF hand motifs. Additionally, we have shown that the coiled-coil domains of MCU interact with MICU1. Identification of these MICU1 and MCU domains provide the critical targets for mitochondrial Ca\(^{2+}\) uptake and potential therapeutic intervention in the pathogenesis of cardiovascular disease states.

**EXPERIMENTAL PROCEDURES**

**Dynamic Protein Flux Assay**
For all studies, MICU1, MCU, and other plasmid constructs were generated from human sequences. HeLa cells were cotransfected with cyto c-GFP and COX8A-mRFP (mitochondrial targeting sequence [MTS]), MICU1-YFP and COX8B-mRFP, or MCU-GFP and COX8B-mRFP plasmid constructs. Forty-eight hours posttransfection, cells were permeabilized with digitonin (0.002% w/v) for 7 min. Permeabilized cells were washed with digitonin-free intracellular-like medium supplemented with 2 mM succinate. After 100 s of baseline recording, permeabilized cells were exposed to either mastoparan (20 µg/ml) or alamethicin (20 µg/ml) at the indicated time points. Confocal images were recorded every 10 s (510 Meta; Carl Zeiss) at 488 and 561 nm excitation using a 40× oil objective. Experiments were performed under physiological conditions with modifications (Kríchek et al., 2004). Cells were recorded using a computer-controlled Axon200B patch-clamp amplifier with a Digitidata 1320A acquisition board (pClamp 10.0 software; Axon Instruments). The ionic composition of the pipette (2, 5, and 100 mM Ca\(^{2+}\)) was chosen based on previous measurements. Mitoplasts with p-orbital morphology were used for patch-clamp recording. Mitoplasts were bathed with a solution containing sodium gluconate (150 mM), KCl (5.4 mM), Ca\(_{\text{Cl}}\) (5 mM), HEPES (10 mM), with a pH of 7.2. The pipette solution contained sodium gluconate (150 mM), NaCl (5 mM), sucrose (135 mM), HEPES (10 mM), and EGTA (1.5 mM), with a pH of 7.2. After formation of G0 seals (pipette resistance 20–35 MΩ), the mitoplasts were ruptured with 200–400 mV pulse varying from 2 to 6 ms duration. Mitoplast capacitance was measured (2.2–3.8 pF). After capacitance compensation, mitoplasts were held at 0 mV and recorded using a voltage clamp (from –160 mV to 80 mV, 120 mV/s).

**Mitoplast Patch-Clamp Recording**
Mitoplast patch-clamp recordings were performed at 30° C as detailed previously with modifications (Kríchek et al., 2004). Currents were recorded using a computer-controlled Axon200B patch-clamp amplifier with a Digitidata 1320A acquisition board (pClamp 10.0 software; Axon Instruments). The ionic composition of the pipette (2, 5, and 100 mM Ca\(^{2+}\)) was chosen based on previous measurements. Mitoplasts with p-orbital morphology were used for patch-clamp recording. Mitoplasts were bathed with a solution containing sodium gluconate (150 mM), KCl (5.4 mM), Ca\(_{\text{Cl}}\) (5 mM), HEPES (10 mM), with a pH of 7.2. The pipette solution contained sodium gluconate (150 mM), NaCl (5 mM), sucrose (135 mM), HEPES (10 mM), and EGTA (1.5 mM), with a pH of 7.2. After formation of G0 seals (pipette resistance 20–35 MΩ), the mitoplasts were ruptured with 200–400 mV pulse varying from 2 to 6 ms duration. Mitoplast capacitance was measured (2.2–3.8 pF). After capacitance compensation, mitoplasts were held at 0 mV and recorded using a voltage clamp (from –160 mV to 80 mV, 120 mV/s).

**Isolation of Endothelial Cells from CVD and Control Subjects**
Primary ECs were isolated from arteries and veins obtained from individuals undergoing surgical intervention for coronary artery disease (CAD)/peripheral artery disease (PAD) at the surgical unit of Temple University Hospital after due ethical clearance. The tissues were collected in standard EC transport medium and transported to the laboratory through cold chain and processed immediately. The tissues were initially washed once with PBS containing 100 U/ml penicillin and 100 µg/ml streptomycin and then cleaned to remove the fibrous portion with sterile forceps. Using a sterile blade, the veins were cut vertically and carefully flipped over. The tissues were cut into small squares of approximate 0.5 cm × 0.5 cm and placed in a 100 mm dish with medium just bathing the tissue pieces. The plates were incubated at 37°C, 5% CO\(_2\). After 1 week, the tissue pieces were removed leaving the individual colonies to develop into a confluent monolayer. Endothelial cell phenotypes were characterized as previously described (Milovanova et al., 2008).

**Endothelial Migration Assay**
Control, CVD-derived ECs, and CVD + MICU1 rescue ECs were seeded at a density of 1 × 10\(^6\) cells/well in 6-well plates overnight for confluent monolayer. A uniform 1.8 mm scratch running the entire length of the well was created using a sterile 200 µl tip. The wells were washed three times with PBS to remove the cell debris and then bathed in 2 ml complete endothelial medium. After 24 hr, cells were washed and fixed with CAMO Quick Stain II as per manufacturer instructions. The wells were photographed at multiple locations using phase contrast microscopy with a 4× objective. Migration was quantified using Image J software (National Institutes of Health); results are expressed as percent gap closure (Craige et al., 2011).

**In Vivo Knockdown of MICU1 and Vascular Integrity Measurement**
To knock down MICU1 in control mice, we used lentivirus carrying MICU1-shRNA. Three daily doses of MICU1-shRNA (1 × 10\(^8\)TU/ml as measured by p24 ELISA and also confirmed by true infectious titer via puromycin resistance in EC culture; 100 µl/mice was delivered using saline as vehicle) lentivirus or...
control lentivirus-negative shRNA were administered intravenously, and MICU1 expression in the endothelial cells, cardiomyocytes, SMCs, and slineocytes were assessed by qRT-PCR after 5 days from the last injection (8 total days). Freshly isolated primary ECs were rapidly subjected to [Ca\(^{2+}\)]\(_{\text{im}}\) and ROS measurement. The vascular integrity studies were performed as described previously (Gandhirajan et al., 2013).

**Statistical Analysis**

Data were expressed as the means ± SE. Statistical significance was evaluated via one-way ANOVA and Student’s t test. A p value of <.05 was considered statistically significant. All experiments were conducted at least three times unless specified. Data were plotted either with GraphPad Prism version 5.0 or with Sigma Plot 11.0 software.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.11.026.

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**REFERENCES**

Aichberger, K.J., Mitterman, I., Reineiner, R., Seiberler, S., Swoboda, I., Spitzer, S., Kopp, T., Stingl, G., Sperr, W.R., Valentin, P., et al. (2005). Hom s 4, an IgE-reactive autoantigen belonging to a new subfamily of calcium-binding proteins, can induce Th cell type 1-mediated autoreactivity. J. Immunol. 175, 1286–1294.

Babcock, D.F., Herrington, J., Goodwin, P.C., Park, Y.B., and Hille, B. (1997). Mitochondrial participation in the intracellular Ca\(^{2+}\) network. J. Cell Biol. 136, 833–844.

Baines, C.P., Kaiser, R.A., Purcell, N.H., Blair, N.S., Osinska, H., Hambleton, M.A., Brunskill, E.W., Sayen, M.R., Gottlieb, R.A., Dorn, G.W., et al. (2005). Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 434, 658–662.

Balaban, R.S. (2009). The role of Ca\(^{2+}\)\(_{\text{cyt}}\) signaling in the coordination of mitochondrial ATP production with cardiac work. Biochim. Biophys. Acta 1787, 1334–1341.

Balaban, R.S., Nemoto, S., and Finkel, T. (2005). Mitochondria, oxidants, and aging. Cell 120, 483–495.

Baughman, J.M., Perocchi, F., Girgis, HS., Plovanich, M., Belcher-Timmel, C.A., Sanac, Y., Bao, X.R., Strittmatter, L., Goldberg, O., Bogorad, R.L., et al. (2011). Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. Nature 476, 341–345.

Bernardi, P. (1999). Mitochondrial transport of cations: channels, exchangers, and permeability transition. Physiol. Rev. 79, 1127–1155.

Berridge, M.J., Bootman, M.D., and Roderick, H.L. (2003). Calcium signalling: dynamics, homeostasis and remodelling. Nat. Rev. Mol. Cell Biol. 4, 517–529.

Cárdenas, C., Miller, R.A., Smith, I., Bu, T., Molgò, J., Müller, M., Vais, H., Cheung, K.H., Yang, J., Parker, I., et al. (2010). Essential regulation of cell bioenergetics by constitutive InsP3 receptor Ca\(^{2+}\)\(_{\text{cyt}}\) transfer to mitochondria. Cell 142, 270–283.

Chaudhuri, D., Sanac, Y., Mootha, V.K., and Clapham, D.E. (2013). MCU encodes the pore conducting mitochondrial calcium currents. eLife 2, e00704.

Clapham, D.E. (2007). Calcium signaling. Cell 131, 1047–1058.

Craig, S.M., Chen, K., Pei, Y., Li, C., Huang, X., Chen, C., Shibata, R., Sato, K., Walsh, K., and Keaney, J.F., Jr. (2011). NADPH oxidase 4 promotes endothelial angiogenesis through endothelial nitric oxide synthase activation. Circulation 124, 731–740.

Csordás, G., Golenár, T., Seifert, E.L., Kamer, K.J., Sanac, Y., Perocchi, F., Moffat, C., Weaver, D., de la Fuente Perez, S., Bogorad, R., et al. (2013). MICU1 controls both the threshold and cooperative activation of the mitochondrial Ca\(^{2+}\)\(_{\text{uniporter}}\). Cell Metab. 17, 976–987.

Davidson, S.M., and Duchen, M.R. (2007). Endothelial mitochondria: contributing to vascular function and disease. Circ. Res. 100, 1128–1141.

De Stefani, D., Raffaello, A., Teardo, E., Szabó, I., and Rizzuto, R. (2011). A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature 476, 336–340.

Denton, R.M., and McCormack, J.G. (1980). The role of calcium in the regulation of mitochondrial metabolism. Biochem. Soc. Trans. 8, 266–268.

Drago, I., Pizzo, P., and Pozzan, T. (2011). After half a centurym mitochondrial calcium in- and efflux machineries reveal themselves. EMBO J. 30, 4119–4125.

Duchen, M.R., Verkhovsky, A., and Mualem, S. (2008). Mitochondria and calcium in health and disease. Cell Calcium 44, 1–5.

Fieni, F., Lee, S.B., Jan, Y.N., and Kirichok, Y. (2012). Activity of the mitochondrial calcium uniporter varies greatly between tissues. Nature communications 3, 1317.

Gandhirajan, R.K., Meng, S., Chandramoorthy, H.C., Malliakan karan, M., Mancarella, S., Gao, H., Kazmierczak, R., Yang, X.F., Houser, S.R., Chen, J., et al. (2013). Blockade of NOX2 and STIM1 signaling limits lipopolysaccharide-induced vascular inflammation. J. Clin. Invest. 123, 887–902.

Gunter, T.E., Gunter, K.K., Sheu, S.S., and Gavin, C.E. (1994). Mitochondrial calcium transport: physiological and pathological relevance. Am. J. Physiol. 267, C313–C339.

Hajnóczky, G., Robb-Gaspers, L.D., Seitz, M.B., and Thomas, A.P. (1995). Decoding of cytosolic calcium oscillations in the mitochondria. Cell 82, 415–424.

Hajnóczky, G., Csordás, D., Das, S., Garcia-Perez, C., Saotome, M., Sinha Roy, S., and Yi, M. (2006). Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca\(^{2+}\)\(_{\text{uptake}}\) in apoptosis. Cell Calcium 40, 553–560.

Hancock, J.F., Paterson, H., and Marshall, C.J. (1990). A polybasid domain or palmitoylation is required in addition to the CAAAX motif to localize p21ras to the plasma membrane. Cell 63, 133–139.

Hansford, R.G. (1994). Physiological role of mitochondrial Ca\(^{2+}\) transport. J. Bioenerg. Biomembr. 26, 495–508.

Heo, W.D., Inoue, T., Park, W.S., Kim, M.L., Park, B.O., Wandless, T.J., and Meyer, T. (2006). PI(3,4,5)P3 and PI(4,5)P2 lipids target proteins with polybasic clusters to the plasma membrane. Science 314, 1458–1461.

Joiner, M.L., Koval, O.M., Li, J., He, B.J., Allamargot, C., Gao, Z., Luzczak, E.D., Hall, D.D., Fink, B.D., Chen, B., et al. (2012). CaMKII determines mitochondrial stress responses in heart. Nature 491, 269–273.

Kirichok, Y., Krapivinsky, G., and Clapham, D.E. (2004). The mitochondrial calcium uniporter is a highly selective ion channel. Nature 427, 360–364.

Kluck, R.M., Estopi, M.D., Perkins, G., Renken, C., Kwon, H., Bossy-Wetzel, E., Goldberg, M., Allen, T., Barber, M.J., Green, D.R., and Newmeyer, D.D. (1999). The pro-apoptotic proteins, Bid and Bax, cause a limited permeabilization of the mitochondrial outer membrane that is enhanced by cytosol. J. Cell Biol. 147, 809–822.

Lemasters, J.J., Theruvath, T.P., Zhong, Z., and Nieminen, A.L. (2009). Mitochondrial calcium and the permeability transition in cell death. Biochim. Biophys. Acta 1787, 1395–1401.

Libby, P., Akawa, M., and Jain, M.K. (2006). Vascular endothelium and atherosclerosis. Handbook Exp. Pharmacol. 2006, 285–306.
Mallilankaraman, K., Cardenas, C., Doonan, P.J., Chandramoorthy, H.C., Irrinki, K.M., Golenar, T., Csordas, G., Madireddi, P., Yang, J., Muller, M., et al. (2012a). MCUR1 is an essential component of mitochondrial Ca\(^{2+}\) uptake that regulates cellular metabolism. Nat. Cell Biol. 14, 1336–1343.

Mallilankaraman, K., Doonan, P., Cardenas, C., Chandramoorthy, H.C., Miller, M., Hoffman, N.E., Gandhirajan, R.K., Molgo, J., Birnbaum, M.J., et al. (2012b). MICU1 is an essential gatekeeper for MCU-mediated mitochondrial Ca\(^{2+}\) uptake that regulates cell survival. Cell 151, 630–644.

McCormack, J.G., Halestrap, A.P., and Denton, R.M. (1990). Role of calcium ions in regulation of mammalian intramitochondrial metabolism. Physiol. Rev. 70, 391–425.

Milovanova, T., Chatterjee, S., Hawkins, B.J., Hong, N., Sorokina, E.M., Debolt, K., Moore, J.S., Madesh, M., and Fisher, A.B. (2008). Caveolae are an essential component of the pathway for endothelial cell signaling associated with abrupt reduction of shear stress. Biochim. Biophys. Acta 1783, 1866–1875.

Mumby, S.M., Heukeroth, R.O., Gordon, J.I., and Gilman, A.G. (1990). G-protein alpha-subunit expression, myristoylation, and membrane association in COS cells. Proc. Natl. Acad. Sci. USA 87, 728–732.

Nicholls, D.G. (1978). The regulation of extramitochondrial free calcium ion concentration by rat liver mitochondria. Biochem. J. 176, 463–474.

Nicholls, D.G. (2005). Mitochondria and calcium signaling. Cell Calcium 38, 311–317.

Orenius, S., Zhivotovsky, B., and Nicotera, P. (2003). Regulation of cell death: the calcium-apoptosis link. Nat. Rev. Mol. Cell Biol. 4, 552–565.

Perocchi, F., Gohil, V.M., Girgis, H.S., Bao, X.R., McCombs, J.E., Palmer, A.E., and Mootha, V.K. (2010). MICU1 encodes a mitochondrial EF hand protein required for Ca\(^{2+}\) uptake. Nature 467, 291–296.

Pfeiffer, D.R., Gudz, T.I., Novgorodov, S.A., and Erdahl, W.L. (1995). The peptide mastoparan is a potent facilitator of the mitochondrial permeability transition. J. Biol. Chem. 270, 4923–4932.

Rizzuto, R., De Stefani, D., Raffaello, A., and Mammucari, C. (2012). Mitochondria as sensors and regulators of calcium signalling. Nat. Rev. Mol. Cell Biol. 13, 566–578.

Santo-Domingo, J., and Demaurex, N. (2010). Calcium uptake mechanisms of mitochondria. Biochim. Biophys. Acta 1797, 907–912.

Soboloff, J., Rothberg, B.S., Madesh, M., and Gill, D.L. (2012). STIM proteins: dynamic calcium signal transducers. Nat. Rev. Mol. Cell Biol. 13, 549–565.

Williams, C.L. (2003). The polybasic region of Ras and Rho family small GTPases: a regulator of protein interactions and membrane association and a site of nuclear localization signal sequences. Cell. Signal. 15, 302–322.

Williams, G.S., Boyman, L., Chikando, A.C., Khairallah, R.J., and Lederer, W.J. (2013). Mitochondrial calcium uptake. Proc. Natl. Acad. Sci. USA 110, 10479–10486.