OPA1 Modulates Mitochondrial Ca\(^{2+}\) Uptake Through ER-Mitochondria Coupling

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Autosomal Dominant Optic Atrophy (ADOA), a disease that causes blindness and other neurological disorders, is linked to OPA1 mutations. OPA1, dependent on its GTPase and GED domains, governs inner mitochondrial membrane (IMM) fusion and cristae organization, which are central to oxidative metabolism. Mitochondrial dynamics and IMM organization have also been implicated in Ca\(^{2+}\) homeostasis and signaling but the specific involvements of OPA1 in Ca\(^{2+}\) dynamics remain to be established. Here we studied the possible outcomes of OPA1 and its ADOA-linked mutations in Ca\(^{2+}\) homeostasis using rescue and overexpression strategies in Opa1-deficient and wild-type murine embryonic fibroblasts (MEFs), respectively and in human ADOA-derived fibroblasts. Patient cells carrying OPA1 GTPase or GED domain mutations also exhibited altered Ca\(^{2+}\) homeostasis, and the mutations associated with lower OPA1 levels displayed closer ER-mitochondria gaps. Furthermore, in Opa1\(^{-/-}\) MEF background, we found that acute expression of OPA1 GTPase mutants but no GED mutants, partially restored cytosolic [Ca\(^{2+}\)]\(_{\text{cyto}}\) needed for a prompt [Ca\(^{2+}\)]\(_{\text{mito}}\) rise. OPA1 mutants’ overexpression in WT MEFs disrupted Ca\(^{2+}\) homeostasis, partially recapitulating the observations in ADOA patient cells. Thus, OPA1 modulates functional ER-mitochondria coupling likely through the OPA1 GED domain in Opa1\(^{-/-}\) MEFs. Therefore, the co-existence of WT and mutant forms of OPA1 in patients promotes an imbalance of Ca\(^{2+}\) homeostasis without a domain-specific effect, likely contributing to the overall ADOA progress.

Keywords: mitochondria, OPA1, ADOA, calcium, endoplasmic reticulum
INTRODUCTION

Mitochondria are compartmentalized and dynamic organelles that undergo multiple membrane reshaping processes (Eisner et al., 2018). Mitochondria reshaping involves mitochondrial fusion, fission, and IMM folding. The GTPase protein, OPA1, is the master regulator of IMM reshaping: controlling IMM fusion, folding, cristae biogenesis, and cristae shape (Liu et al., 2009; Song et al., 2009; Varanita et al., 2015; Gylysou et al., 2016; Hu et al., 2020). Mitochondrial cristae host OXPHOS supercomplexes and support mitochondrial and cellular metabolism (Vogel et al., 2006; Cogliati et al., 2013). The multiple functions of OPA1 rely on several domains, which include the GTPase domain and the GTPase effector domain (GED) (Landes et al., 2010).

Ca^{2+} controls OXPHOS and in turn, ATP production by activating different dehydrogenases and supporting pyruvate supply inside the mitochondria (Denton, 1990; Hajnóczky et al., 1995; Jouaville et al., 1999). Ca^{2+} is principally stored at the Endoplasmic Reticulum (ER), and its release to the cytosol is mediated through the activation of the IP_{3} receptor (IP3R) or the Ryanodine receptor (Berridge, 2016). Mitochondria can physically interact with the ER through multiple protein tethers to facilitate the local Ca^{2+} transfer from the ER to mitochondria, and cytosolic Ca^{2+} (Ca_{cyt}^{2+}) clearance (Rizzuto et al., 1998; Csdás et al., 1999; Csdás et al., 2006). Mitochondrial Ca^{2+} (Ca_{mito}^{2+}) uptake is determined by: 1) the Voltage-dependent Anion Selective Channels (VDACs) and Mitochondrial Calcium Uniporter complex (mtCU), located at the outer mitochondrial membrane (OMM) and IMM, respectively; and 2) ΔΨ_{m}, which is the main component of the electrochemical gradient acting as the driving force (Gunter and Sheu, 2009; Rizzuto et al., 2012; Hajnóczky et al., 2014). In addition, mitochondrial morphology is a potential modulator of Ca_{mito}^{2+} homeostasis that needs further investigation (Szabadkai et al., 2004; Szabadkai et al., 2006; Kowaltowski et al., 2019).

Pathogenic mutations in OPA1 trigger Autosomal Dominant Optic Atrophy (ADOA, MIM#165500) which causes blindness due to Retinal Ganglion Cells (RGCs) death (Delettre et al., 2000; Olichon et al., 2003; Amati-Bonneau et al., 2008). OPA1 dysfunction induces IMM fusion abolition, IMM rearrangement, cristae shape disturbance and metabolic disruption (Song et al., 2009; Patten et al., 2014). The axons of mice RGCs expressing ADOA mutants, show drastic mitochondrial depletion, due to autophagosome accumulation at axonal hillocks in a AMPK-dependent manner (Zaninello et al., 2020).

The relevance of OPA1 in Ca^{2+} homeostasis has been described in the literature with many discrepancies between different groups. Knockdown of Opa1 in RGCs caused altered Ca_{mito}^{2+} clearance upon ER Ca^{2+} (Ca_{ER}^{2+}) release and excitotoxicity, suggesting that OPA1 may have a role in Ca^{2+} homeostasis and disease progress (Dayanithi et al., 2010; Kushnareva et al., 2013). However, silencing of OPA1 in HeLa and H295R cell lines induced enhanced Ca_{mito}^{2+} uptake upon IP3R activation despite low ΔΨ_{m} and mitochondrial morphology changes (Fülöp et al., 2011). In contrast, another group showed that OPA1 knockdown in HeLa cells displayed diminished Ca_{mito}^{2+} uptake upon IP3R stimulation with serious defects on Ca_{mito}^{2+} retention capacity, associated with a decrease in cristae number (Kushnareva et al., 2013). In a different study, ADOA-derived fibroblasts from a family carrying the same OPA1 GTPase mutation showed an enhanced Ca_{mito}^{2+} uptake upon IP3R stimulation but with a wide variety of responses between patients (Fülöp et al., 2015). And, expression of ADOA-causing mutants in mice RGCs and nematode motoneurons, resulted in augmented Ca_{mito}^{2+} levels (Zaninello et al., 2021). Yet, over 400 different pathogenic OPA1 variants have been described in the Leiden Open Variation Database, LOVD (Delettre et al., 2019) or ClinVar Database (Landrum et al., 2018) which can affect different domains such as the GTPase and GED domain among others. Finally, it is clear that OPA1 loss alters Ca^{2+} homeostasis, but the mechanisms involved still remain elusive.

Ca^{2+} uptake across the IMM is mediated by the mtCU complex. The protein core of this highly regulated complex is composed of the mitochondrial Ca^{2+} uniporter (MCU), the essential MCU regulator (EMRE), and the dominant-negative MCU subunit (MCUb) (Baughman et al., 2011; De Stefani et al., 2011; Raffaello et al., 2013; Sancak et al., 2013). The MCU activity and Ca^{2+} threshold are controlled by the mitochondrial calcium uniporter proteins (MICU1/2/3) (Perocchi et al., 2010; Mallilankaraman et al., 2012; Csdás et al., 2013; Kamer et al., 2017; Patron et al., 2019). An interaction between OPA1 and the MCU-MICU1 complex has been recently described (Herkenne et al., 2020). Also, OPA1 interacts with the mitochondrial cristae and contact site organizing system (MICOS) complex and MICU1, suggesting a role for MICU1 on cristae junction regulation (Gottschalk et al., 2019; Tomar et al., 2019). These data suggest that the role of OPA1 on Ca^{2+} homeostasis may involve mechanisms beyond its fusion activity.

High-resolution imaging experiments have shown that cristae are dynamic (Kondadi et al., 2020) and they move throughout the mitochondria in an OPA1-dependent manner (Gottschalk et al., 2018). Cristae localized close to ER-mitochondria contacts decelerate upon IP3R stimulation probably to support ER-mitochondria Ca^{2+} transfer (Gottschalk et al., 2018), reinforcing the potential role of OPA1 on Ca_{mito}^{2+} signaling.

In this work, we studied the role of OPA1 and its disease causing mutations in Ca_{mito}^{2+} responses upon local or bulk Ca_{cyt}^{2+} signaling. We show that OPA1 is necessary to regulate ER-to-mitochondria Ca^{2+} transfer. We also demonstrate that OPA1 GTPase and GED, which are known to cause ADOA when pathogenic mutants are present, differentially alter Ca_{mito}^{2+} homeostasis, with the GED domain playing a key role in ER-to-mitochondria functional coupling. In addition, the coexistence of all studied mutant and wild-type (WT) forms of OPA1 indistinctly disrupts Ca^{2+} homeostasis, potentially contributing to ADOA disease progression.

RESULTS

OPA1 is Required for Cytosolic and Mitochondrial Ca^{2+} Homeostasis

To test the relevance of OPA1 in Ca^{2+} homeostasis, we first used MEFs in which the mitochondrial matrix-targeted Ca^{2+}-sensitive
FIGURE 1 | OPA1 is required for cytosolic and mitochondrial Ca²⁺ homeostasis. (A) ER-dependent mitochondrial Ca²⁺ uptake, schematic experimental design. WT, Opa1⁻/⁻ or acute rescued Opa1⁻/⁻ MEF Cells with OPA1 WT isoform 1 expressing mtRCaMP, and the muscarinic receptor 3 (M3R), were incubated with Fura2-AM (2 µM) for 20 min at room temperature and stimulated with 200 µM of Carbachol (CCh) to induce the release of Ca²⁺ from the ER to the cytosol via activation of IP3R (B). The graph shows mean traces of [Ca²⁺]cyto expressed in nM (WT, n/equals/3/64; preparations/cells; Opa1⁻/⁻, n/equals/3/87; Opa1⁻/⁻ + OPA1 WT, n/equals/3/100) (C) Mean traces of [Ca²⁺]mito. (D,E) Maximal [Ca²⁺]cyto and [Ca²⁺]mito amplitude upon agonist stimulation. (F) The graph shows mean traces of [Ca²⁺]mito uptake vs IP3R-induced (Continued)
protein, mtRCaMP was expressed to monitor the \([Ca^{2+}]_{\text{mito}}\) simultaneously with \([Ca^{2+}]_{\text{cyto}}\) tracked by a \(Ca^{2+}\) sensing dye, Fura2-AM. The cells were also transfected with the muscarinic type 3 receptor (M3R) to allow Carbachol (CCh)-induced \([Ca^{2+}]_{\text{cyto}}\) transients through activation of IP3R-mediated \(Ca^{2+}\) mobilization from the ER (Figure 1A).

First, we explored the differences in the \(Ca^{2+}\) signaling between WT and Opa1\(^{−/−}\) cells. Our data showed lower resting \([Ca^{2+}]_{\text{cyto}}\) levels (50.2 ± 2.9 nM) in Opa1\(^{−/−}\) cells compared with WT cells (127.8 ± 7.8 nM), and upon the acute rescue with the human OPA1 isoform 1 (OPA1), we found a partial restoration of the baseline (83.6 ± 5.2 nM, Supplementary Figure S1A). After CCh stimulation, we observed lower maximal \([Ca^{2+}]_{\text{mem}}\) amplitude in Opa1\(^{−/−}\) cells (333.6 ± 21.4 nM) compared with WT cells (775.9 ± 47.6 nM). Moreover, the exogenous expression of OPA1 partially rescued the \([Ca^{2+}]_{\text{cyto}}\) signal (544.6 ± 25.8 nM, Figures 1B,D).

The resting \([Ca^{2+}]_{\text{mito}}\) was elevated in Opa1\(^{−/−}\) cells. This was also detected by an alternative \(Ca^{2+}\) sensor, mCepiPA3. In addition, OPA1 rescue further increased resting \([Ca^{2+}]_{\text{mito}}\) suggesting that acute restoration of OPA1 to mitochondria is insufficient to lower basal \([Ca^{2+}]_{\text{mito}}\) to WT levels (Supplementary Figures S1B–E). The baseline-normalized agonist-stimulated \([Ca^{2+}]_{\text{mito}}\) transients showed no differences between WT and Opa1\(^{−/−}\) cells, whereas the OPA1-rescued MEFs showed an increased \([Ca^{2+}]_{\text{mito}}\) (Figures 1C,E). Interestingly, after reaching the maximum \([Ca^{2+}]_{\text{mito}}\) amplitude, Opa1\(^{−/−}\) cells exhibited faster decay kinetics than WT cells, however, this decay in the \([Ca^{2+}]_{\text{mito}}\) is not rescued by acute expression of OPA1.

To further explore the correlation between \([Ca^{2+}]_{\text{mito}}\) and IP3-linked \(Ca^{2+}\) release, the two variables were plotted against each other (Figure 1F). The graph shows that Opa1\(^{−/−}\) cells needed less \([Ca^{2+}]_{\text{cyto}}\) to induce \(Ca^{2+}\)mito release than WT cells, which can be partially rescued by the expression of OPA1 (Figure 1G). To test the possibility of mitochondrial \(Ca^{2+}\) uptake capacity saturation, we plotted the maximal \([Ca^{2+}]_{\text{mito}}\) vs the maximal \([Ca^{2+}]_{\text{cyto}}\) in single WT cells. As shown in Supplementary Figure S1F, there is a positive correlation between the \([Ca^{2+}]_{\text{cyto}}\) peak and the \([Ca^{2+}]_{\text{mito}}\) peak, confirming that the mitochondrial \(Ca^{2+}\) uptake or the \(Ca^{2+}\) sensor was not saturated in the range of the responses. Moreover, lower \([Ca^{2+}]_{\text{cyto}}\) was needed to induce maximal \([Ca^{2+}]_{\text{mito}}\) (Supplementary Figure 1G). In summary, the \([Ca^{2+}]_{\text{cyto}}\) records indicate lesser ER \(Ca^{2+}\) content/release, whereas the \([Ca^{2+}]_{\text{mito}}\) - \([Ca^{2+}]_{\text{cyto}}\) relationship seems to support more effective decoding of the IP3R-mediated \(Ca^{2+}\) release by mitochondrial \(Ca^{2+}\) uptake in Opa1\(^{−}\) cells than that in the control cells.

To evaluate the ER \(Ca^{2+}\) content/release, in a \(Ca^{2+}\)-free extracellular medium, we stimulated the cells with Thapsigargin (2 µM) to discharge ER \(Ca^{2+}\) by blocking the ER-\(Ca^{2+}\) re-uptake through the Sarcoendoplasmic Reticulum Calcium-ATPase (SERCA). To follow the \(Ca^{2+}\) that the mitochondria face upon \(Ca^{2+}\)ER release, the cells were transfected with an outer mitochondrial membrane-targeted \(Ca^{2+}\) sensor (OMMRCaMP). Both \([Ca^{2+}]_{\text{cyto}}\) and \([Ca^{2+}]_{\text{OMM}}\) responses showed a decreased \(Ca^{2+}\) release from ER (Supplementary Figures S1H–K), suggesting that the free \(Ca^{2+}\)ER content is attenuated in the Opa1\(^{−}\) cells and this is not reverted by the acute expression of OPA1.

To further test the ER \(Ca^{2+}\) content, in a \(Ca^{2+}\)-free extracellular medium, we first stimulated the cells with CCh to induce IP3R-mediated release of \(Ca^{2+}\) from the ER, and then, we added Thapsigargin to discharge the residual ER \(Ca^{2+}\) (Supplementary Figure S1L). The \([Ca^{2+}]_{\text{cyto}}\) responses confirmed that ER \(Ca^{2+}\) content is reduced in the Opa1\(^{−/−}\) cells than that in the WT. Acute expression of OPA1 failed to rescue the ER \(Ca^{2+}\) content. (Supplementary Figures S1M,N).

During CCh stimulation, mostly local ER-to-mitochondria \(Ca^{2+}\) transfer mediates the \([Ca^{2+}]_{\text{mito}}\) rise. To test if the \([Ca^{2+}]_{\text{mito}}\) increase induced by a bulk \([Ca^{2+}]_{\text{cyto}}\) increase is also altered in the Opa1\(^{−}\)-deficient cells, Store Operated Calcium Entry (SOCE) was induced by adding 1 mM CaCl\(_2\) after the ER \(Ca^{2+}\) depletion (Figure 1H; Supplementary Figure S1L). After SOCE induction, Opa1\(^{−}\) cells showed an augmented \([Ca^{2+}]_{\text{cyto}}\) transient amplitude (790.7 ± 44.2 nM) compared with WT cells (629.4 ± 44.5 nM), and this effect was completely rescued upon OPA1 expression (525.1 ± 40.0 nM, Figures 1I,K). The corresponding \([Ca^{2+}]_{\text{mito}}\) responses were slightly but significantly augmented in Opa1\(^{−}\) cells compared with WT but similar to that in the OPA1 rescued cells (Figures 1J,L). However, plotting \([Ca^{2+}]_{\text{mito}}\) vs. \([Ca^{2+}]_{\text{cyto}}\) revealed higher \([Ca^{2+}]_{\text{cyto}}\) requirement to induce \([Ca^{2+}]_{\text{mito}}\) in Opa1\(^{−}\) cells than WT or rescued cells (Figures 1M,N; Supplementary Figure S1O). Thus, Opa1\(^{−}\) cells have an advantage in the mitochondrial response to IP3R-mediated \(Ca^{2+}\) release from the ER but they have a disadvantage in the response to the SOCE-mediated global \([Ca^{2+}]_{\text{cyto}}\) increase.
To test if some of the differences between WT, Opa1<sup>−/−</sup>, and rescued cells might result from a difference in the driving force for the mitochondrial Ca<sup>2+</sup> uptake, we studied mitochondrial membrane potential (ΔΨ<sub>mito</sub>) by a potentiometric dye, TMRE. Consistent with Fülöp et al. (Fülöp et al., 2011), we found that Opa1<sup>−/−</sup> cells exhibited lower resting ΔΨ<sub>mito</sub> than WT cells, which was partially rescued by OPA1 (Figure 1O). This might be a reason for the relatively weak [Ca<sup>2+</sup>]<sub>mito</sub> response by the Opa1<sup>−/−</sup> cells to the enhanced SOCE.

We next tested if a difference in the mitochondrial mass or mtCU abundance/composition might explain the differential [Ca<sup>2+</sup>]<sub>mito</sub> responses in the Opa1<sup>−/−</sup> cells and the controls. We found that both, Opa1<sup>−/−</sup> and OPA1 rescued cells exhibited elevated levels of mitochondrial mass markers (Supplementary Figures S2A,B). We also found that Opa1<sup>−/−</sup> MEFs showed no changes in the main mtCU components after mitochondrial mass normalization (Supplementary Figures S2C,D).

Finally, we tested the ER-mitochondrial spatial relationship and mitochondrial ultrastructure as a potential source of the differences in the [Ca<sup>2+</sup>]<sub>mito</sub> responses by transmission electron microscopy (TEM). Our results showed IMM topology perturbation and cristae compartmentalization in the Opa1<sup>−/−</sup> MEFS, as previously described (Frezza et al., 2006; Olichon et al., 2007a). Furthermore, we found significantly closer ER-mitochondria gaps in Opa1<sup>−/−</sup> cells compared with WT cells (Opa1<sup>−/−</sup>: 15.71 ± 0.41 nm vs. WT: 17.92 ± 0.38 nm) (Figure 1P). To test the OPA1-specificity of our findings, we generated Opa1<sup>−/−</sup> + OPA1 WT stable MEF cells (Supplementary Figure S2E) and tested the ER-mitochondrial distance. We found increased ER-mitochondria distance in OPA1 rescued cells compared with Opa1<sup>−/−</sup> cells (Opa1<sup>−/−</sup> + OPA1: 17.81 ± 0.67 nm vs. Opa1<sup>−/−</sup>: 14.98 ± 0.72) (Figure 1Q).

Thus, we conclude that the lack of Opa1 causes multiple changes in the Ca<sup>2+</sup><sub>cyto</sub> and Ca<sup>2+</sup><sub>mito</sub> homeostasis including an attenuated ER Ca<sup>2+</sup> storage and enhanced SOCE. Still, the Ca<sup>2+</sup><sub>mito</sub> uptake response to IP3R-mediated Ca<sup>2+</sup> release is enhanced, likely because of closer physical proximity between mitochondria and the ER.

**Mitochondrial Ca<sup>2+</sup> Homeostasis, ΔΨ<sub>m</sub>, and ER-Mitochondria Contacts Are Altered in ADOA-Derived Patient Cells Carrying OPA1 Domain-specific Mutations**

We wanted to address if ADOA patient-derived fibroblasts carrying domain-specific heterozygous OPA1 mutations replicate the defects in Ca<sup>2+</sup> homeostasis and ER-mitochondria distance Opa1<sup>−/−</sup> cells exhibited. Fibroblasts carrying specific mutations at the GTPase domain included OPA1 c.870+5G>A, with a deletion of the Exon8, and c.889C>T with an early stop codon at GTPase domain. Both patients presented a severe form of the disease, involving other symptoms besides optic nerve atrophy, known as ADOA+ (MIM# 125250). Also, we studied patients carrying specific mutations in GED: OPA1 c.2713C>T leading to a stop codon and loss of the GED domain, and c.2818+5G>A with a deletion of GED coding exon 27. These patients presented symptoms restricted to the eye or plain ADOA (MIM#165500) (Figure 2A). Western blot analysis revealed that patients’ cells carrying c.889C>T, c.870+5G>A, and c.2818+5G>A mutations, exhibited lower OPA1 protein levels compared with Control cells or the c.2713C>T mutant (Figure 2B; Supplementary Figure S3A).

To evaluate intracellular Ca<sup>2+</sup> signaling, we first measured resting [Ca<sup>2+</sup>]<sub>mito</sub>. Our results showed that patients’ cells have unaltered resting [Ca<sup>2+</sup>]<sub>cyto</sub> (Figures 2C,D; Supplementary Figure S3B). Analysis of Ca<sup>2+</sup><sub>mito</sub> showed no differences in [Ca<sup>2+</sup>]<sub>mito</sub> resting in patients’ cells carrying GTPase mutants. However, the GED mutant c.2713C>T showed elevated resting [Ca<sup>2+</sup>]<sub>mito</sub> compared with control patient cells (Supplementary Figure S3C).

Then, we used histamine as an agonist to induce Ca<sup>2+</sup> release from the ER through activation of the IP3R. Data in Figures 2C,D show that the patients’ cells displayed comparable maximal [Ca<sup>2+</sup>]<sub>cyto</sub> transient amplitude than control individuals. Cells carrying GTPase mutations showed no significant changes in [Ca<sup>2+</sup>]<sub>mito</sub> rise; however, cells carrying the GED mutant c.2713C>T displayed lower [Ca<sup>2+</sup>]<sub>mito</sub> increase compared with control cells (Figures 2E,F). [Ca<sup>2+</sup>]<sub>mito</sub> vs. [Ca<sup>2+</sup>]<sub>cyto</sub> chart showed that all the ADOA patient’s cells had a leftward shift tendency in this relationship compared to control cells (Figures 2G,H; Supplementary Figure S3D).

Next, we evaluated the resting ΔΨ<sub>mito</sub> in ADOA-derived cells. Fibroblast carrying GTPase mutants showed no significant changes, but GED mutant c.2713C>T exhibited a higher resting ΔΨ<sub>mito</sub> compared with control cells, which may be a factor in the elevated resting [Ca<sup>2+</sup>]<sub>mito</sub> levels and therefore, reduced [Ca<sup>2+</sup>]<sub>mito</sub> transients amplitude (Figure 2I).

Finally, we tested the distance between the ER and the mitochondria. EM analysis of ADOA-derived fibroblasts revealed that cells carrying the GTPase mutants c.889C>T, c.870+5G>A and the GED mutant c.2818+5G>A exhibited significantly closer ER-mitochondria apposition, compared with control cells (Skin Control: 20.24 ± 0.35 nm, OPA1 c.889C>T 16.6 ± 0.35 nm, SkM Control: 20.3 ± 0.39 nm, OPA1 c.870+5G>A 17.93 ± 0.49 nm, OPA1 c.2818+5G>A 18.09 ± 0.38 nm). Yet, we found no differences between control and GED mutant c.2713C>T carrying cells (Figure 2J; Supplementary Figure S3E). Strikingly, all the samples that exhibited closer ER-mitochondria contacts also displayed lower OPA1 protein levels (Figure 2B).

In summary, ADOA-derived fibroblasts showed no defects on [Ca<sup>2+</sup>]<sub>cyto</sub> transients unlike Opa1<sup>−/−</sup> cells. However, we found that all the studied mutants needed lesser Ca<sup>2+</sup><sub>cyto</sub> to trigger a Ca<sup>2+</sup><sub>mito</sub> rise, consistent with the observations in Opa1<sup>−/−</sup> cells. Interestingly, the patients’ cells showed a correlation between OPA1 protein levels and ER-mitochondria distance. Thus, despite the distinct severities of ADOA disease, caused by GTPase and GED mutants, both kinds of aberrant proteins lead to similar Ca<sup>2+</sup> homeostasis dysregulation.

**OPA1 GED Domain Determines Functional ER-to-Mitochondrial Coupling**

Given that patients’ cells might carry genetic or environmental adaptations, we next studied the domain-specific effects of OPA1 mutants in an Opa1<sup>−/−</sup> background. For this, we generated ADOA-causing OPA1 GTPase and GED domain-specific
FIGURE 2 | Mitochondrial Ca$^{2+}$ homeostasis, mitochondrial membrane potential, and ER-mitochondria distance are altered in cells from ADOA patients carrying OPA1 domain-specific mutations. (A) OPA1 disease-related mutations, protein product prediction, type of mutation, and clinical ADOA phenotype, evaluated in this study. (B) Western blot analysis of OPA1 protein abundance of the skin or skeletal muscle-derived fibroblasts from ADOA patient’s cells. (C) Skin or skeletal muscle ADOA-derived fibroblast expressing mtRCaMP were loaded with Fura2-AM (2 μM) for 20 min at room temperature. To induce the release of Ca$^{2+}$ through IP3R activation, Histamine 100 μM was used. The graph show [Ca$^{2+}$]$_{cyto}$ expressed as Fura2-AM 340/380 mean ratio values (Skin Control, n = 3/12; preparations/cells; Skin OPA1 c.889C>T, n = 3/18; SkM Control, n = 5/17, SkM OPA1 c.870+5G>A, n = 7/21; SkM OPA1 c.2713C>T, n = 4/19). (D) The bar chart shows maximal [Ca$^{2+}$]$_{cyto}$ amplitude upon agonist stimulation. (E) Mean traces of Skin or skeletal muscle ADOA-derived fibroblast [Ca$^{2+}$]$_{mito}$ upon Histamine stimulation. (F) The bar chart shows maximal [Ca$^{2+}$]$_{mito}$ uptake upon agonist stimulation expressed in F/F0 to mtRCaMP. (G) The graph shows mean traces of [Ca$^{2+}$]$_{mito}$ uptake vs IP3R-induced [Ca$^{2+}$]$_{cyto}$ release. (H) [Ca$^{2+}$]$_{mito}$ at which WT, Opa1$^{-/-}$, and +OPA1 WT shows 1.4 fold increase in [Ca$^{2+}$]$_{mito}$ upon agonist stimulation. (I) Skin or skeletal muscle fibroblasts from ADOA-patients were loaded with TMRM 10 nM and Fura2-AM. Incubation and stimulation were performed as described above. At the end of the experiment, FCCP 10 μM was added to dissipate me membrane potential. The bar chart shows resting Δψm. (Skin Control, n = 3/25; preparations/cells; Skin OPA1 c.889C>T, n = 3/18; SkM Control, n = 5/25, SkM OPA1 c.870+5G>A, n = 3/26; SkM OPA1 c.2713C>T, n = 3/28; (J) ER-mitochondria distance analysis for all mitochondria with ER contact (Continued)
mutants, matching the patients’ genotype (Supplementary Figure S4A). We observed that different mutants show distinct protein levels, consistent with our observation in the patients’ cells (Figure 2A). Particularly, mutants carrying splicing defects, such as OPA1 c.870+5G>A and c.2818+5G>A, display lower protein levels than WT OPA1, suggesting defects in protein stability.

Our data showed that the acute expression of WT OPA1 rescues decreased resting [Ca2+]cyto levels shown by Opa1−/− cells; nevertheless, the expression of each ADOA-causing OPA1 mutants displayed resting [Ca2+]cyto levels comparable to Opa1−/− cells. These data suggest that OPA1 integrity is critical to support a mitochondrial role in the Ca2+cyto balance under non-stimulated conditions (Figures 3A–B, Supplementary Figure S4B). The analysis of Cm−/−mito showed that all studied OPA1 GED mutants exhibited increased resting [Ca2+]mito (Supplementary Figures S4C–E). Consistently, we observed the same effect in the Opa1−/− cells and the OPA1 GED mutation c.2713C>T patient cells (Supplementary Figures S1A, S3C, respectively), suggesting that the GED region, and not the GTPase domain, may play a specific role in the regulation of [Ca2+]mito resting levels.

Upon CCh stimulation, both WT and OPA1 GTPase mutants rescued the maximal [Ca2+]cyto rise; whereas, GED domain mutants showed no rescue (Figures 3A–C). In terms of maximal [Ca2+]mito transient amplitude triggered by CCh, neither the acute expression of WT OPA1 nor the OPA1 mutants, showed a significant difference compared to Opa1−/− cells (Figures 3D–F). This might be a consequence of a chronic absence of Opa1 or alternatively, more than one OPA1 isoform is needed to restore Cm−/−mito homeostasis, as it has been demonstrated for mitochondrial network morphology (Del Dotto et al., 2017).

We next studied the correlation between ER-associated [Ca2+]mito and [Ca2+]cyto responses. Our data showed that as in Opa1−/− background, the expression of OPA1 GED mutants, displayed a leftward shift in [Ca2+]cyto, needed to induce a [Ca2+]mito rise compared with WT or GTPase rescued conditions (Figures 3G–I). Moreover, the levels of [Ca2+]cyto needed to generate the maximal [Ca2+]mito were rescued by the OPA1 GTPase mutants, while OPA1 mutants lacking GED displayed only a mild rescue (Supplementary Figure S4F). Thus, our data suggest that Ca2+ transfer to the mitochondria from local Ca2+cyto is enhanced when the GED region or the entire protein is missing. However, this asseveration doesn’t totally match with the GTPase mutant c.889C>T, which expresses a truncated protein form due to a stop codon (Figure 2A) and showed a behavior comparable to the GTPase mutants rather than the GED mutants.

To test the OPA1 domain-specific effects on the Cm−/−mito rise prompted by bulk Ca2+cyto increases, we turned to SOCE. Upon SOCE induction, the augmented [Ca2+]cyto transients of the Opa1−/− cells were restored by WT OPA1. All the mutants also displayed [Ca2+]cyto transients comparable with WT OPA1 (Figures 3J–L). However, no significant effect was observed on [Ca2+]mito upon SOCE induction in Opa1−/− or rescued cells (Figures 3M–O).

A two-variable chart was plotted to explore the role of bulk Ca2+mito entry on ER-independent Ca2+mito uptake. The graphs show that upon [Ca2+]cyto increase, Opa1−/− cells exhibited a rightward shift in [Ca2+]mito needed to induce [Ca2+]mito rise, compared with the GTPase or GED mutants (Figures 3P–R). Moreover, Opa1−/− mitochondria required 790.7 ± 44.2 nM of [Ca2+]cyto to reach the maximal [Ca2+]mito and this was completely restored with the inclusion of either OPA1 or the mutants, requiring lower [Ca2+]cyto to reach the maximal [Ca2+]mito (Supplementary Figure S4G).

Next, we measured resting ΔΨm to test if the driving force of Ca2+ uptake is altered upon the expression of some of the OPA1 mutants. No change in resting ΔΨm was detected in the GTPase mutant c.870+5G>A (Figure 3S). We found the GED mutant c.2713C>T exhibited an augmented resting ΔΨm compared with OPA1 WT rescue (Figure 3S), as we observed in the patient cells harboring the same mutant (Figure 2I). Thus, although WT and GTPase OPA1 mutants can restore ER-to-mitochondrial Ca2+ transfer, this seems independent of the cation driving force evoked by ΔΨm. Finally, Mcu protein levels were unaltered by the acute expression of neither OPA1 GTPase nor GED mutants (Figure 3T).

Thus, OPA1 GED region plays a critical role in ER-mitochondrial Ca2+ transfer, and this effect is independent of resting ΔΨm, OPA1, or Mcu protein levels. Moreover, Cm−/−mito uptake induced by bulk Ca2+cyto, was restored by OPA1 or the mutants suggesting that the sole physical presence of the protein is sufficient to support this process, in a domain-specific independent manner.

Overexpression of OPA1 Mutants impairs Ca2+ Homeostasis

Considering the heterozygosity of the ADOA patients carrying OPA1 mutations, we tested if the introduction of OPA1 mutants, in the presence of native Opal in the WT MEF background, disrupts normal Ca2+ homeostasis like what was observed in the patient cells.

Our results showed that acute expression of OPA1 mutants did not alter resting [Ca2+]cyto (Supplementary Figure S4H). Interestingly, the GTPase mutant c.1334G>A exhibited a high resting [Ca2+]mito when compared with WT OPA1 overexpression (Supplementary Figures S4I–K).

Upon CCh stimulation, we found a decreased maximal [Ca2+]cyto amplitude in the GTPase mutant c.1334G>A when...
Figure 3 | OPA1 GED domain is required for effective ER-to-mitochondrial Ca²⁺ transfer. (A,B) ER-dependent Ca²⁺ release. Opa1⁻/⁻ MEF cells expressing OPA1 mutants, mIP3CaMP, and the muscarinic receptor 3 (MSR), were incubated with Fura2-AM as described, and stimulated with 200 µM of Carbachol (CCh) to induce the release of Ca²⁺ from the ER to the cytosol via activation of mIP3R. Graph shows mean traces of [Ca²⁺]cyt expressed in nM. (A) Shows mean traces of GTPase mutants and (B) shows mean traces of GED mutants of [Ca²⁺]cyt transients, respectively (Opa1⁻/⁻ MEF, n = 3/87; Opa1⁻/⁻ + OPA1 WT, n = 3/100; Opa1⁻/⁻ + OPA1 c.870+5G>A, n = 3/55; Opa1⁻/⁻ + OPA1 c.889C>T, n = 3/55; Opa1⁻/⁻ + OPA1 c.1334G>A, n = 3/35; Opa1⁻/⁻ + OPA1 c.2713C>T, n = 3/69; Opa1⁻/⁻ + OPA1 c.2713C>T, n = 3/109; Opa1⁻/⁻ + OPA1 c.2818+5G>A, n = 3/55). (C) The...
is compared with WT OPA1 overexpression (Figures 4A–C). The same mutant exhibited an elevated maximal \([\text{Ca}^{2+}]_{\text{mito}}\) transient amplitude upon agonist stimulation (Figures 4D–F). In addition, GED mutant c.870+5G>A exhibited a reduced maximal \([\text{Ca}^{2+}]_{\text{cyto}}\) amplitude after agonist stimulation compared to WT OPA1 overexpression (Figure 4C). Moreover, both GED mutants c.2713C>T and c.2818+5G>A displayed a reduced \([\text{Ca}^{2+}]_{\text{mito}}\) (Figure 4F). The same reduction in \([\text{Ca}^{2+}]_{\text{mito}}\) was observed in the ADOA patient’s cells carrying the GED mutant c.2713C>T.

Correlative study of \([\text{Ca}^{2+}]_{\text{mito}}\) and \([\text{Ca}^{2+}]_{\text{cyto}}\) transients showed that overexpression of OPA1 WT displayed no alteration in the \([\text{Ca}^{2+}]_{\text{cyto}}\) needed to trigger a \([\text{Ca}^{2+}]_{\text{mito}}\) rise. In contrast, the acute expression of most GTPase or GED mutants, prompted a leftward shift in \([\text{Ca}^{2+}]_{\text{cyto}}\) needed to induce \([\text{Ca}^{2+}]_{\text{mito}}\) rise upon agonist stimulation (Figures 4G–I). Consistently, as opposed to WT OPA1 overexpression, the inclusion of GTPase or GED mutants lowered the maximal \([\text{Ca}^{2+}]_{\text{cyto}}\) needed to evoke maximal \([\text{Ca}^{2+}]_{\text{mito}}\) (Supplementary Figure 5A-L).

Evaluation of \(\Delta \psi_{\text{m}}\) revealed that acute overexpression of OPA1 WT decreased resting \(\Delta \psi_{\text{m}}\) (Figure 4J). Yet, neither GTPase mutant c.870+5G>A nor GED mutant c.2713C>T overexpression altered resting \(\Delta \psi_{\text{m}}\) in a WT MEF background. (Figure 4J). Finally, we found no differences in Mcu protein levels caused by WT or mutant OPA1 overexpression (Figure 4K).

Thus, acute expression of ADOA-causing OPA1 mutants in the presence of endogenous Opa1 causes a dominant-negative phenotype independent of the domain affected by the specific mutation possibly contributing to ADOA disease progression (Figure 4L; Supplementary Table S1).

In different cell types, the absence of OPA1 has shown diverse results. In cultured RGCs, the principal cells affected by OPA1 mutants causing DOA (Votrubá et al., 1998), silencing of Opa1 augmented Ca\(^{2+}\) transients (Dayanithi et al., 2010; Kushnareva et al., 2013). Interestingly, RGCs expressing OPA1 ADOA-causing mutants, show elevated Ca\(^{2+}\) transients (Zanimelli et al., 2021). However, proopiomelanocortin neurons devoid of Opa1 show unaltered Ca\(^{2+}\)\(_{\text{cyto}}\) transients, and reduced Ca\(^{2+}\)\(_{\text{mito}}\) responses (Gómez-Valadés et al., 2021). Finally, adult cardiomyocytes of Opa1\(^{-/-}\) mice displayed lower Ca\(^{2+}\)\(_{\text{cyto}}\) amplitude (Chen et al., 2012; Le Page et al., 2016) or enhanced Ca\(^{2+}\)\(_{\text{mito}}\) uptake (Piquereau et al., 2012). These studies suggest that OPA1 affects intracellular Ca\(^{2+}\) homeostasis, possibly, in a cell-type specific manner.

Our data in Opa1\(^{-/-}\) cells exhibited alterations in cytosolic, ER and mitochondrial Ca\(^{2+}\) homeostasis. Resting Ca\(^{2+}\)\(_{\text{cyto}}\) was reduced in Opa1\(^{-/-}\), which could be explained by a decreased influx or increased efflux of Ca\(^{2+}\). The former is unlikely because a higher influx was detected upon SOCE, whereas, the latter is not consistent with our \(\Delta \psi_{\text{m}}\) data, which suggests lower ATP levels in these cells. Also, CCh-induced Ca\(^{2+}\)\(_{\text{cyto}}\) transient amplitude was decreased in Opa1\(^{-/-}\) cells, caused by lower ER content, suggesting that chronic absence of Opa1 leads to an adaptation in ER Ca\(^{2+}\) homeostasis, that could involve IP3R or SERCA levels or activity alterations. Moreover, resting and maximal \([\text{Ca}^{2+}]_{\text{mito}}\) were augmented despite low resting \(\Delta \psi_{\text{m}}\) during local Ca\(^{2+}\) transfer. This observation is consistent with previous reports in OPA1 silenced intact HeLa and H295R cells using a genetically encoded Ca\(^{2+}\) sensor (Fülöp et al., 2011). Conversely, another group reported OPA1 silencing decreases maximal \([\text{Ca}^{2+}]_{\text{mito}}\) amplitude upon Histamine stimulation, using Rhod-2 as a Ca\(^{2+}\) sensor in intact cells (Kushnareva et al., 2013). These opposite results can be attributed to the different technical approaches. The mitochondria-specific nature of \([\text{Ca}^{2+}]_{\text{mito}}\) transients studied by genetic encoded Ca\(^{2+}\)-sensitive proteins, provides strong pieces of evidence to confirm that both acute (Fülöp et al., 2011), and chronic absence of OPA1 studied in this work lead to augmented Ca\(^{2+}\)\(_{\text{mito}}\) uptake. Hence, our study strengthens the idea of OPA1 as a key molecular modulator in ER-to-mitochondria Ca\(^{2+}\) transfer.

DISCUSSION

We showed here that cells with low levels of Opa1 exhibit closer ER-mitochondria apposition, likely resulting in a more efficient ER-to-mitochondria Ca\(^{2+}\) transfer. This occurs without changes in the mtCU components or mitochondrial mass, and despite the reduced resting \(\Delta \psi_{\text{m}}\) presented by the Opa1-deficient cells. In an Opa1\(^{-/-}\) background, ADOA-causing mutants located in OPA1 GED region perturbed ER-dependent Ca\(^{2+}\)\(_{\text{cyto}}\) transients and Ca\(^{2+}\)\(_{\text{mito}}\) uptake. In WT background, OPA1 mutants evoke a dominant-negative phenotype independent of the domain affected by the specific mutation possibly contributing to ADOA disease progression (Figure 4L; Supplementary Table S1).
Overexpression of ADOA-causing OPA1 mutants impairs ER-to mitochondrial Ca$^{2+}$ transfer.  

(A,B) WT MEF cells expressing OPA1 mutants, mitoRCaMP, and the muscarinic receptor 3 (M3R), were incubated with Fura2-AM and stimulated with 200 µM of Carbachol (CCh) to induce the release of Ca$^{2+}$ from the ER to the cytosol via activation of IP3R. Graph shows mean traces of [Ca$^{2+}$]cyto expressed in nM, in WT MEF expressing GTPase and (B) in GED mutants (WT MEF, n = 3/64; WT + OPA1 WT, n = 3/58; WT + OPA1 c.870+5G>A, n = 3/59; WT + OPA1 c.889C>T, n = 3/48; WT + OPA1 c.1334G>A, n = 3/51; WT + OPA1 c.2708delTTAG, n = 3/47; WT + OPA1 c.2713C>T, n = 3/49; WT + OPA1 c.2818+5G>A, n = 3/42).  

(C) Bar chart shows maximal [Ca$^{2+}$]cyto amplitude upon agonist stimulation.  

(D,E) The (Continued)
The reduced \([\text{Ca}^{2+}]_{\text{cyto}}\) needed to induce a \([\text{Ca}^{2+}]_{\text{mito}}\) rise by \(\text{Opa1}^{-/-}\) cells likely reflects enhanced ER-to-mitochondrion privileged communication, which might be caused by the increased proximity between both organelles. Yet, this was independent of resting mitochondrial \(\Delta\psi_{\text{m}}\) and mtCU protein levels. RNA-seq data has shown that mtCU components expression levels were unaltered in Opal KO lymphocytes (Corrado et al., 2021). In addition, silencing of Micu1 increases resting matrix \([\text{Ca}^{2+}]_{\text{mito}}\) (Mallilankaraman et al., 2012; Liu et al., 2021). Interestingly, upon IP3R activation, cristae stop their motion but no maximal \([\text{Ca}^{2+}]_{\text{mito}}\) was augmented. Elevated resting \([\text{Ca}^{2+}]_{\text{cyto}}\), given that neither GTPase nor GED mutants altered resting \([\text{Ca}^{2+}]_{\text{cyto}}\). The GTPase domain was not required to restore \([\text{Ca}^{2+}]_{\text{cyto}}\) needed to induce \([\text{Ca}^{2+}]_{\text{mito}}\) rise, suggesting that OPA1 GED domain could be relevant during ER-mitochondria contact reorganization.

To isolate the domain-specific mutants‘ effect we performed in vitro studies in Opal \(+/−\) background. We found that the integrity of the whole OPA1 protein is necessary to restore \([\text{Ca}^{2+}]_{\text{cyto}}\), given that neither GTPase nor GED mutants displayed an enhanced \([\text{Ca}^{2+}]_{\text{mito}}\) uptake rate (Fülöp et al., 2015). However, the GTPase mutants explored in this study, both in patient’s cells and acutely expressed in MEFs, displayed no changes in \([\text{Ca}^{2+}]_{\text{mito}}\). Interestingly, the previous study found a high dispersion between patients carrying the same mutation (Fülöp et al., 2015), suggesting that part of the effect could be related to environmental adaptations or the patient’s genetic background.

Finally, ADOA is a dominant disease, most of the patients are heterozygous OPA1 mutant, with only a few reports of patients with homozygous OPA1 mutations (MIM #605290). The inclusion of OPA1 GTPase or GED mutants in WT MEF cells did not alter \([\text{Ca}^{2+}]_{\text{cyto}}\) whereas, resting but no maximal \([\text{Ca}^{2+}]_{\text{mito}}\) was augmented. Elevated resting \([\text{Ca}^{2+}]_{\text{mito}}\), also found in the patient’s cells harboring the OPA1 GED mutant c.2713C>T, suggesting that this effect could be specific for this domain. In addition, the GED mutants did not restore the \([\text{Ca}^{2+}]_{\text{cyto}}\) needed to induce \([\text{Ca}^{2+}]_{\text{mito}}\) rise, suggesting that the OPA1 GED domain is essential for the maintenance of \([\text{Ca}^{2+}]_{\text{mito}}\) uptake mediated by ER-mitochondria Ca\(^{2+}\) transfer. OPA1 GED domain is a predicted coiled-coil domain (Akepati et al., 2008); however, no molecular partner, except for SIRT3, has been linked to physically interact with this domain (Samant et al., 2014). Strikingly, GED mutant’s expression restored bulk \([\text{Ca}^{2+}]_{\text{cyto}}\), needed to induce \([\text{Ca}^{2+}]_{\text{mito}}\) rise, proposing that OPA1 GED domain could be relevant during ER-mitochondria functional tethering through a, so far, unknown molecular partner.

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mitochondria distance, suggesting that OPA1 integrity and levels are necessary for physiological intracellular Ca\(^{2+}\) regulation and ER-mitochondria Ca\(^{2+}\) transfer. Thus, the cellular models studied here, with patient cells and the acute expression of OPA1 mutants in a WT background, all point towards a dominant-negative Ca\(^{2+}\) homeostasis phenotype, independent of the involved domain.

In conclusion, our study provides new evidence on a central role for OPA1 in Ca\(^{2+}\) homeostasis and in determining functional ER-mitochondrial coupling, with the GED region playing a fundamental role in stabilizing the efficiency of inter-organelle Ca\(^{2+}\) transfer. The co-existence of WT and ADOA-related mutants could perturb ER-to-mitochondrial communication, providing a mechanism for disease progression in patients affected with ADOA disease.

**MATERIALS AND METHODS**

**Cell Culture**

Experiments were performed in fibroblasts derived from ADOA patients and control individuals or WT and Opa1 KO Mouse embryonic fibroblasts (MEFs) (provided by David Chan). Patient-derived cells were provided by Newcastle Research Biobank for Rare and Neuromuscular Diseases, based on a Material Transfer Agreement with Pontificia Universidad Católica de Chile, and were maintained following the institutional biosecurity and bioethics protocols. All the cells were cultured in high glucose Dulbecco-Eagle modified medium containing sodium pyruvate (DMEM, Gibco Cat#12800017) and supplemented with 10% of fetal bovine serum (FBS), 2 mM glutamine, and 100 U/ml penicillin, and 100 μg/ml streptomycin in humidified air (5% CO\(_2\)) at 37°C. Given that human myoblasts grow slowly, skeletal muscle-derived fibroblasts were generated from myoblast samples. Briefly, the patient’s derived myoblasts were cultured in Skeletal Muscle Cell Growth Medium (PromoCell) and plated in collagen I-coated dishes. Cells were trypsinized and pre-plated in an uncoated dish for 30 min to separate fibroblasts from myoblasts, where the former adhere to the uncoated dish while the latter are resuspended and placed aside. The patient’s fibroblasts were used between passages 3–9. All the cells were tested for mycoplasma contamination regularly using the following protocol (Young et al., 2010).

**Cell Transfection**

Cells were plated on glass 25 mm coverslips and then transfected with specific constructs using Lipofectamine 2,000–3,000 (Invitrogen). MEF cells were transfected pCCEY plasmid encoding for human OPA1 isoform 1. OPA1 GTPase or GED mutants were built in the same backbone. For mitochondrial matrix calcium analysis, we used mitochondrial matrix-targeted Ca\(^{2+}\)-sensitive proteins mtRCaMP (K\(_d\) ~ 1 μM) and CEP1A3mt (K\(_d\) ~ 10 μM). The transfection was performed with OPTI-mem (Thermofisher) or Transfectagro (Corning) and lipofectamine 2,000 or 3,000 (Invitrogen, Cat#11668019 or Cat#L300015, respectively) according to the manufacturer’s protocol. For optimal expression, the cells were grown for 48 h after transfection. One μg of DNA per plasmid per 35 mm dish was used for each experiment.

**Ca\(^{2+}\) Measurement**

MEFs were transfected with the mtRCaMP, the muscarinic receptor type 3 (M3R), and OPA1 mutants. Forty-eight hours after transfection the cells were incubated in a serum-free extracellular medium (ECM: 121 mM NaCl, 5 mM NaHCO3, 10 mM Na-HEPES, 4.6 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 10 mM glucose, pH7.4) containing 2% BSA and loaded with Fura2-AM (2 μM) in presence of 0.0003% Pluronic F-127 and 100 μM sulfinpyrazone for 15 min at room temperature. Cells were washed once with ECM containing 2% BSA, and recorded in ECM containing 0.25% BSA, and transferred to the thermostated stage (37°C) of the microscope. Cells were stimulated by Carbachol (CCh) 200 nM to activate the M3R, and subsequently, the IP3R, to induce Ca\(^{2+}\) release from the ER. For SOCE, after Fura2-AM incubation, cells were washed once with a Ca\(^{2+}\) free buffer. Imaging was performed in a Ca\(^{2+}\)-free ECM containing 0.25% BSA. Cells were stimulated by CCh 200 nM to activate the IP3R and induce Ca\(^{2+}\) release from the ER and recorded for 150s, then the cells were stimulated with Thapsigargin 2 μg to deplete the ER of Ca\(^{2+}\) and recorded for 300s, and finally, 1 mM of Ca\(^{2+}\) was added to induce SOCE. Images were acquired using an ImagEM EM-CCD camera (Hamamatsu) fitted to an Olympus IX81 microscope with LED source (Lambda TLED+, Sutter Instruments). The configuration for each fluorescent probe or protein was as follow: Fura2-AM was recorded using 340–380 nm excitation; mtRCaMP was recorded with a 577 nm excitation filter; CEP1A3mt was recorded using 485 nm excitation filter (Chroma, customized 59022 UV filters), using dual-band dichroic and emission filters (Chroma, 59022m dual-band filters). Image collection frequency, 1 Hz. Calibration of the Fura2-AM signal was carried out at the end of each measurement, adding 1 mM CaCl\(_2\), followed by 10 nM EGTA/Tris, pH 8.5. The Fura2-AM ratios were calibrated in terms of nM [Ca\(^{2+}\)]\(_{cyto}\) using the following formula:

$$[	ext{Ca}^{2+}]_{	ext{cyto}} \text{ (nM)} = K_d \times \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \times \frac{[\text{F}380_{\text{max}}]}{F380_{\text{min}}}$$

Where Fura2-AM K\(_d\) was 224 nM, R is ratio 340/380 nm, R\(_{\text{min}}\) is the ratio 340/380 after EGTA addition, R\(_{\text{max}}\) is the ratio 340/380 nm after 1 mM CaCl\(_2\) addition, F\(_{\text{max}}\) is the maximum 380 nm fluorescence upon EGTA addition. F\(_{\text{min}}\) is the minimum 380 nm fluorescence upon 1 mM CaCl\(_2\) addition. Maximal [Ca\(^{2+}\)]\(_{\text{mito}}\) uptake was calculated as F\(_{\text{max}}\)/F\(_0\) where F\(_{\text{max}}\) is the maximum fluorescence intensity and F\(_0\) is the mean resting fluorescence intensity before IP3R stimulation or SOCE protocol induction.

For [Ca\(^{2+}\)]\(_{\text{mito}}\) vs. [Ca\(^{2+}\)]\(_{\text{cyto}}\) analysis we plotted the mean trace curve from basal to maximal [Ca\(^{2+}\)]\(_{\text{cyto}}\) in X-axis, with regards to the same time points corresponding to [Ca\(^{2+}\)]\(_{\text{mito}}\) relative fluorescence from basal to maximal [Ca\(^{2+}\)]\(_{\text{cyto}}\) in the Y-axis. For maximal IP3R-induced [Ca\(^{2+}\)]\(_{\text{cyto}}\) vs maximal [Ca\(^{2+}\)]\(_{\text{mito}}\) uptake we used the maximal [Ca\(^{2+}\)]\(_{\text{cyto}}\) ± SEM in the X-axis, and the maximal [Ca\(^{2+}\)]\(_{\text{mito}}\) uptake ± SEM, in the Y-axis. To quantify the changes in the [Ca\(^{2+}\)]\(_{\text{mito}}\) vs. [Ca\(^{2+}\)]\(_{\text{cyto}}\) we consider considered the [Ca\(^{2+}\)]\(_{\text{cyto}}\) at the moment of a 2-fold increase in
Western Blot Analysis
Cells were cultured to 70–80% of confluence, and frozen. Upon thawing, a membrane-rich lysate was generated by RIPA buffer supplemented with protease and phosphatase inhibitors; 30 µg of total protein extracts were loaded into 8% or 4–12% gradient SDS-PAGE gel and transferred to PVDF membranes. Membranes were blocked with 5% milk in 0.1% TBS-Tween for 1 h at RT, followed by overnight incubation with primary antibody prepared in 5% milk or 3% BSA in 0.1% TBS-Tween. Secondary antibodies were visualized with enhanced chemiluminescent substrates (ECL, SuperSignal West Dura or SuperSignal West Femto, Thermo Scientific). For MICU1, MICU2, and EMRE, were used IR LI-COR Bioscience secondary antibodies. Densitometry was performed using ImageStudio software (LI-COR Bioscience). Antibodies used in this study: OPA1(1:1,000, BD Pharmigen #Cat 612607), MICU1 (1:500, Sigma #Cat HPA37497), MICU2 and EMRE (1:1,000, Bethyl) MCU (1:1,000, Cell signaling #Cat14997, 1:500 Sigma-Aldrich #Cat HPA016480), mtHSP70 (1:1,000 Invitrogen, #Cat MA3-028), TIM23 (1:1,000, BD Pharmigen #Cat 611-222), TOM20 (1:1,000, Proteintech #Cat 11802-1-AP), TUBULIN (1:1,000 Cell Signaling #Cat 2144S), GAPDH (1:5,000, Proteintech #Cat 60004-1-lg).

OPA1 Domain-specific Mutations Plasmid Construction
The pCCYE plasmid containing human OPA1 isoform 1 WT was kindly donated by Guy Lenaers. Mutagenic OPA1 variants were designed using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) and following the manufacturer’s protocol. The actual generation of specific mutations was confirmed by sequencing using the ABI PRISM 3500xl Applied Biosystems (FONDEQUIP ELM150077) at Pontificia Universidad Católica de Chile or sequenced at Macrogen inc., Seoul, South Korea. pCCYE plasmid encoding GTPase mutant OPA1 c.899G>A (G300E) and GED mutant OPA1 c.2708delTTAG was kindly donated by Guy Lenaers and used in previous studies (Olichon et al., 2007b; Eisner et al., 2014). For primers’ details, see Table 1.

Stable Cell Line Generation
Opal1−/− MEFs cells were transfected as described above with a lentiviral plasmid containing the human OPA1 isoform 1 and a puromycin resistance cassette (pLenti-OPA1). Transfected cells were selected with Puromycin 2 µg/ml to obtain a polyclonal stable cell line. The cells were grown in growth media described above in presence of Puromycin 2 µg/ml. The pLenti-OPA1 was obtained from VectorBuilder.
**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

**AUTHOR CONTRIBUTIONS**

BC-S and VE conceived the project. BC-S, GH, and VE designed the experiments. BC-S performed the research. BC-S, GH, and VE analyzed the data. PYWM provided the patient’s cells. BC-S, DA, and ME-A designed and prepared the plasmids. DL and JM prepared the Opa1−/− + OPA1 stable cell line. JM analyzed the TEM images obtained with the Opa1−/− + OPA1 stable cell line. BC-S and VE wrote the paper with comments from all authors. All the authors read and approve the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.774108/full#supplementary-material
**Supplementary Figure 4** | (A) Western blot quantification of OPA1 levels. (B) Skin or skeletal muscle ADCA-derived fibroblasts expressing mtRCAmp were loaded with Fura2-AM (2 μM) for 20 min at room temperature. To induce the release of Ca2+ through IP3R activation, Histamine 100 μM was used. The bar chart shows resting [Ca2+]cytosol. (G) The bar chart shows resting [Ca2+]cytosol. (D) Maximal [Ca2+]cytosol uptake vs Maximal IP3R-induced [Ca2+]cytosol amplitude upon agonist stimulation. (E) TEM representative images of ER-mitochondria contacts from ADCA-derived patient cells. ER is pseudo-colored in blue. Data are mean ± SEM. Error bars represent SEM. *p < 0.01, **p < 0.001 vs. Control condition. (F) Maximal [Ca2+]cytosol uptake vs Maximal [Ca2+]cytosol amplitude induced upon agonist stimulation. (G) Maximal [Ca2+]cytosol uptake vs Maximal [Ca2+]cytosol upon SOCE induction. (H) WT MEF cells expressing OPA1 mutants, mtRCAmp, and the muscarinic receptor 3 (M3R), were incubated with [Ca2+]cytosol expressed in nM. The bar chart shows resting [Ca2+]cytosol. (I) Graphs show mean traces of [Ca2+]cytosol expressed in arbitrary fluorescence units, evoked by GTPase mutants. (J) Graphs show mean traces of [Ca2+]cytosol expressed in arbitrary fluorescence units, evoked by GED mutants. (K) The bar chart shows resting [Ca2+]cytosol. (L) Maximal [Ca2+]cytosol vs Maximal [Ca2+]cytosol amplitude induced upon agonist stimulation. Data are mean ± SEM. Error bars represent SEM. *p < 0.05, **p < 0.01 vs OP toxins−/− or WT condition. #p < 0.05, ##p < 0.01 vs OPα−/− + OPA1 WT condition. The blue color is indicative of GTPase mutants and the red color for GED mutants.

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