The ATPase Inhibitory Factor 1 (IF_1) regulates the expression of the mitochondrial Ca^{2+} uniporter (MCU) via the AMPK/CREB pathway

Danilo Faccenda[1], Giulia Gorini[2], Adam Jones[1], Claire Thornton[1,3], Alessandra Baracca[2], Giancarlo Solaini[2], Michelangelo Campanella[1,4]

[1]Department of Comparative Biomedical Sciences, The Royal Veterinary College, University of London, 4 Royal College Street, NW1 0TU, London, United Kingdom; [2]Department of Biomedical and Neuromotor Sciences, Laboratory of Biochemistry and Mitochondrial Pathophysiology, University of Bologna, Via Irnerio 48, 40126, Bologna, Italy; [3]Department of Perinatal Imaging and Health, St Thomas' Campus, King's College London, 163 Lambeth Palace Road, SE1 7EH, London, United Kingdom. [4]Department of Cell and Developmental Biology, Consortium for Mitochondrial Research (CfMR), University College London, Gower Street, WC1E 6BT, London, United Kingdom.

Correspondence: Michelangelo Campanella, Department of Comparative Biomedical Sciences, The Royal Veterinary College, University of London, Royal College Street, NW1 0TU, London, United Kingdom, mcampanella@rvc.ac.uk

Word count (excluding Title, Acknowledgments and References): 25997 characters (with spaces).

Number of figures: 4.

Abstract

Mitochondria are key regulators of intracellular Ca^{2+} homeostasis. The tight control of mitochondrial Ca^{2+} entry and cycling is therefore critical to avoid alterations underlying the development of severe pathological conditions, including cancer and neruodegeneration. The mitochondrial Ca^{2+} uniporter (MCU) complex is acknowledged as the mediator of Ca^{2+} influx into mitochondria. Nevertheless, the mechanisms governing its expression and activity remain ill-defined. Here, we show that the mitochondrial protein ATPase inhibitory factor 1 (IF_1) regulates MCU-dependent Ca^{2+} uptake via a retrograde-signal-mediated regulatory mechanism. IF_1 is a key mediator of mitochondrial energy preservation, and its loss correlates with higher mitochondrial membrane potential ($\Delta\Psi_m$), increased generation of reactive oxygen species (ROS), alterations in the inner mitochondrial structure and engagement of mitochondrial apoptosis under stress. Here we show that, in IF_1-depleted HeLa cells, mitochondria sequester Ca^{2+} more efficiently following intracellular mobilization. This event is paired with decreased cellular ATP levels, inducing the activation of the AMPK/CREB pathway and the consequent upregulation of the MCU complex. Interestingly, down-regulation of the mitochondrial inner membrane metalloprotease OMA1, which promotes OPA1 processing and cristae remodelling in response to stress, prevents cellular ATP depletion and restores physiological levels of MCU and mitochondrial Ca^{2+} entry. We propose that impaired mitochondrial bioenergetic function induced by loss of IF_1 underlies mitochondrial dysfunction exacerbated through excessive Ca^{2+} uptake via MCU.

Key Words: mitochondria, Ca^{2+}, MCU, IF_1 and AMPK

Introduction

Calcium ions (Ca^{2+}) are universal intracellular messengers that control a diverse array of cellular functions, including cell metabolism (1), migration (2), division (3) and death (4). Other physiological roles of Ca^{2+} comprise the regulation of muscle contraction, nerve contractility, hormone secretion (5) and immune response (6). The versatility of Ca^{2+} signalling depends on Ca^{2+} interaction with a wide range of intracellular sensors and transducers through varied spatiotemporal dynamics. This implies that cells has to maintain their cytosolic free Ca^{2+} concentration at very low levels (~100 nM) to allow for the sudden, rapid and compartmentalized Ca^{2+} transients. Intracellular Ca^{2+} signalling is therefore allowed by the presence of plasma membrane Ca^{2+} pumps and intracellular Ca^{2+} stores. The former constantly transport the dication out of the cell to maintain its 20000-fold gradient across the plasmalemma (7), while the latter rapidly uptake or release Ca^{2+} to quench or initiate Ca^{2+} signals. The presence of intracellular stores also enable the functional organization of Ca^{2+} transients in signalling microdomains. Together with the endoplasmic reticulum, which is the main intracellular site of Ca^{2+} storage, mitochondria have a central role as dynamic Ca^{2+} buffers. Mitochondrial Ca^{2+} handling is a fundamental feature of mitochondrial physiology. By sequestering and releasing Ca^{2+}, mitochondria act as hubs for Ca^{2+} storage and buffering, thereby tuning the local Ca^{2+} availability and shaping cellular Ca^{2+} signals (8-10). The movements of Ca^{2+} across mitochondria mainly depend on the activity of the mitochondrial Ca^{2+} uniporter (MCU), which regulates the influx pathway, and the Na^{+}/Ca^{2+}(NCLX) and H^{+}/Ca^{2+} (mHCX) exchangers, which instead control Ca^{2+} efflux. Even though the molecular determinants of these cation channels have been extensively characterized (11,12), the mechanisms regulating their function have not been yet fully unravelled. Moreover, in recent years, a vast body of evidence has emerged linking defective mitochondrial Ca^{2+} handling to an ever expanding number of pathological conditions, including neurodegenerative diseases (13-17). Hence, furthering the current understanding of the mechanisms that regulate mitochondrial Ca^{2+} homeostasis is crucial to the development of advanced, targeted treatment options.

Mitochondrial Ca^{2+}-storage capacity is highly correlated with the bioenergetic state of mitochondria. Mitochondrial Ca^{2+}-uptake is driven by both high mitochondrial membrane potential ($\Delta\Psi_m$) and low intramatrix concentration of the dication (18).

The extent of the latter is indirectly regulated by the rate of respiration and oxidative phosphorylation. By establishing the proton-motive force across the mitochondrial inner membrane, they directly control the electrical gradient favouring Ca^{2+} entry, and indirectly set the chemical gradient through the activity of NCLX and mHCX. On the other hand, Ca^{2+} is a major regulator of ATP production. Changes in mitochondrial matrix Ca^{2+} concentration modulate the activity of various mitochondrial dehydrogenases and the F_1F_o-ATP synthase, thereby promoting the TCA cycle and ATP synthesis (19,20).

Ca^{2+} is therefore a key regulator of the activity state of the F_1F_o-ATP synthase, in combination with the mitochondrial protein ATPase inhibitory factor 1 (IF_1) (21). The latter primarely acts as a modulator of the F_1F_o-ATP synthase, inhibiting ATP hydrolysis during conditions of impaired $\Delta\Psi_m$, when the enzymatic complex acts in reverse. Hence, IF_1 prevents excessive ATP hydrolysis, cell energy imbalance and death under ischaemia/hypoxia (22,23). Recent studies, including works from our group, have revealed the existence of a larger plethora of functions for IF_1, and outlined the pathological implications of its deregulation (24-28).

In particular, we recently uncovered a role for the ATPase inhibitory factor 1 (IF_1) in evasion of apoptosis, which contributes to the pro-tumorigenic function of the protein (29,30). This oncogenic mechanism occurs through OPA1-dependent maintenance of cristae shape and preservation of redox balance. Loss of IF_1 activity in HeLa cells promotes stress-dependent OPA1 degradation, causing the presence of mitochondria with abnormal cristae morphology, which ultimately impairs cell metabolism predisposing to cell death. To further investigate into the impact of IF_1 on mitochondrial function, we here studied the role of the inhibitory protein in down-regulating mitochondrial Ca^{2+} buffering capacity. Our interest stemmed from the notion that alterations in mitochondrial Ca^{2+} handling can lead to pathological cell death through mitochondrial swelling and permeability transition (18), which are both counteracted when IF_1 is over-expressed (30). Moreover, mitochondrial Ca^{2+} buffering capacity appears to be impaired in cells devoid of OPA1 (31,32), which accumulate Ca^{2+} at a greater extent.

The present study uncovers a novel function for IF_1 in the control of mitochondrial Ca^{2+} homeostasis, and describes the existence of an AMPK-mediated adaptive mechanism

through which inefficient respiration and ATP synthesis, in the absence of IF_1, drive MCU upregulation to preserve mitochondrial function. The pathway is promoted by the activation of OMA1, a metalloendopeptidase involved in mitochondrial inner membrane remodelling. This further proves the tight correlation between mitochondrial function and structure, and the crucial role played by IF_1 in their regulation.

Materials and Methods

Cell lines, culture and transfection

HeLa cervical adenocarcinoma cells stably transduced with an shRNA targeting human IF_1 mRNA (IF_1 KD) or a non-silencing shRNA (Scrm) were cultured according to standard tissue culture protocols as described in Supporting Information.

TEM

Control and IF_1 KD HeLa were fixed and processed for transmission electron microscopy as previously described (33). Images were acquired using a JEOL JEM-1010 transmission electron microscope and analysed as reported in Supporting Information.

Live-cell imaging

Live-cell imaging of cytosolic free Ca^{2+} transients was carried using the fluorescent Ca^{2+} indicator Fura-2, AM. Mitochondrial Ca^{2+} uptake was evaluated by using the genetically encoded, mitochondria-targeted ratiometric fluorescent probe 2mtGCaMP6m (34). A detailed description of the imaging and analysis methods is reported in Supporting Information.

Western blotting

Cells were harvested 24 hours after cell seeding or 48 hours after transfection and processed as described in Supporting Information. In all experiments, protein samples were separated on a polyacrylamide gel and then transferred onto a PVDF membrane. After incubation with the appropriate primary and HRP-conjugated secondary antibodies, protein bands were detected using chemiluminescence and quantified via densitometry. For a list of antibodies and incubation conditions please refer to Supporting Information.

Quantification of total cellular ATP content

Relative intracellular ATP levels were determined 24 hours after cell seeding or 48 hours from transfection using a chemiluminescent detection assay as reported in Supporting Information.

Real-time quantitative PCR

Total cellular RNA was extracted 24 hours after cell seeding and 1 µg RNA was retrotranscribed for each sample as described in Supporting Information. Real-time

quantitative PCR was performed loading 1:20 cDNA sample per reaction and analysed as reported in Supporting Information.

Results

IF_1 loss increases intracristal space and Ca^{2+} accumulation

We previously reported that stable knock-down of IF_1 in HeLa cells causes abnormal topology of the mitochondrial inner membrane, which is characterized by a reduced folding and the appearance of distorted, horseshoe-shaped cristae (33). Interestingly, mitochondrial cristae in IF_1 KD cells also have a significant increase in width, which is particularly noticeable at the crista junctions (**Figure 1A,B**). While this observation goes in line with the reduced stability of OPA1 observed in cells lacking IF_1 function (33), it might also underlie alteration in molecular diffusion from the intermembrane space into the mitochondrial matrix (35). To test the hypothesis that knocking-down IF_1 alters mitochondrial Ca^{2+} handling and favours the accumulation of Ca^{2+} into the mitochondrial matrix, control and stable IF_1 KD HeLa cells were co-transfected with mitochondria-targeted GCaMP6m (2mtGCaMP6m) and red fluorescent protein (mtRFP), to follow mitochondrial Ca^{2+} uptake over time after the administration of the calcium mobilising agonist histamine. Immediately after histamine administration, transfected cells responded with a transient increase in 2mtGCaMP6m fluorescence, which corresponded to a mitochondrial Ca^{2+} spark (**Figure 1C**). Despite the absence of variations in the basal intramitochondrial Ca^{2+} levels (**Figure 1E**), the amplitude of the mitochondrial fluorescence increase was around 50% higher in IF_1 KD cells (**Figure 1D**), indicating a higher mitochondrial Ca^{2+} uptake capacity. Nevertheless, we did not observe any difference between the two cell types in both amplitude and dynamics of histamine-induced intracellular Ca^{2+} rise, which was monitored by staining cells with Fura-2 AM (**Figure 1F,G**). Histamine induces intracellular Ca^{2+} mobilization by activating H1 histamine receptors and causing both emptying of intracellular ER stores via phospholipase C and capacitative Ca^{2+} influx from the extracellular environment (36,37). This two-phase increase response might explain why the augmented mitochondrial Ca^{2+} uptake capacity of IF_1 KD cells was not matched by changes in cytosolic buffering. Therefore, to measure the Ca^{2+} uptake capacity of mitochondria following ER emptying alone, and highlight variations in the coupling between the two organelles, we then used the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin to promote the passive release of Ca^{2+} from the ER. Confirming the previous results, IF_1 KD cells exhibited a 50% increase in mitochondrial accumulation of Ca^{2+}, the levels of which also stayed elevated for longer

due to inhibition of ER Ca^{2+} reuptake (**Figure 1H,I**). Moreover, IF_1 loss also disturbed the whole-cell Ca^{2+} homeostasis, as confirmed by the lower levels of cytosolic Ca^{2+} accumulation (**Figure 1J,K**).

IF_1 loss induces MCU upregulation through AMPK-dependent CREB activation

We then questioned what might cause the increase in mitochondrial Ca^{2+} uptake in cells down-regulated for IF_1. In our cell model, the reduction in IF_1 activity has been correlated with higher mitochondrial membrane potential and impaired ATP synthesis at resting conditions (38-40). Therefore, these conditions might enhance mitochondrial Ca^{2+} entry in IF_1 KD cells as an adaptive mechanism to maximize ATP production by enhancing oxidative phosphorylation (41-43). Alternatively, the increase in mitochondrial Ca^{2+} uptake might be sustained by increased activity of the mitochondrial calcium uniporter (MCU) complex. Hence, we analysed via Western blotting the protein levels of the pore forming subunit of the complex, MCU itself. Quite unexpectedly, MCU expression was greatly increased in IF_1 KD cells (**Figure 2A,B**) independently of mitochondrial mass, as quantified via immunodetection of ATPB and VDAC (**Figure 2D,E**). To further investigate the mechanism of MCU-dependent increase in mitochondrial Ca^{2+} entry in IF_1 KD cells, we looked at changes in mRNA and protein levels of the different components of the MCU complex. In accordance with the Western blotting data, IF_1 KD cells showed a nearly 1.6 increase in MCU transcript levels, which was matched by an approximately 1.5-fold increase in the expression of all other subunits with the exception of a 2-fold increase in that of EMRE (**Figure 2F**). By measuring the expression ratio of the MCU complex subunits to MCU itself, it also appears that in IF_1 KD cells the stoichiometry of the complex is maintained (**Figure 2G**). This might indicate that IF_1 KD cells have a higher mitochondrial content of fully functional, non-constitutively active, MCU complex. To investigate further into the mechanism leading to MCU upregulation in IF_1 KD cells, we then checked the expression and activation status of cAMP response element-binding protein (CREB), a known transcriptional regulator of MCU transcription (44,45). Among the CREB activating pathways, we focused on 5' AMP-activated protein kinase (AMPK)-mediated signalling (46,47). First, increased levels of AMPK activation has recently been shown in the heart of IF_1 KO mice (48).

Secondly, our data indicated that knock-down of IF_1 attenuates intracellular Ca^{2+} signalling, therefore ruling out the initiation of CREB-dependent MCU transcription

following a rise in cytosolic free Ca^{2+} levels (44,49). To confirm the involvement of an AMPK-mediated, CREB-dependent mechanism of MCU expression, we quantified the total and phosphorylated protein levels for both factors (**Figure 2H-O**). Our results showed a remarkable increase in the degree of both AMPKα and CREB phosphorylation when IF_1 is down-regulated (2- and 1.5-fold increase, respectively; **Figure 2J,N**). Of notice, while the total levels of CREB were unchanged between the two cell lines, AMPKα expression was significantly higher in IF_1 KD cells (**Figure 2I,M**). This indicates that IF_1 depletion promotes AMPK activity via upregulation of the protein rather than by augmenting the rate of its phosphorylation, which is not affected by changes in IF_1 expression (**Figure 2K**). Collectively, our data suggest that IF_1 might control MCU expression in resting cells by limiting AMPK-mediated CREB activation, which is instead enhanced when the protein is down-regulated (**Figure 2O**). Further confirming this hypothesis, treatment with the adenylate cyclase activator forskolin, which triggers CREB-mediated gene transcription, significantly increased MCU expression and protein levels in control cells (**Figure 2Q-S**). This result validate MCU as a CREB-dependent genes in our cell model. Considering that AMPK is rapidly activated when ATP production decreases, and that a reduction in intracellular ATP levels has been documented in IF_1 knock-down HeLa cells (38), we compared the total cellular ATP levels between the two cell lines to confirm the loss of ATP-dependent increase in AMPK activity. As expected, IF_1 KD cells showed a nearly 40% decrease in cellular ATP levels (**Figure 2P**).

Down-regulation of OMA1 limits MCU upregulation and mitochondrial Ca^{2+} uptake in IF_1 KD cells

Even though the role of the MCU complex as master regulator of mitochondrial Ca^{2+} entry is aknowlegded, it has also been proposed that the geometry of crista junctions, which is altered when IF_1 is down-regulated (**Figure 1**), might control mitochondrial Ca^{2+} fluxes (35). Moreover, Ca^{2+} uptake appears to be facilitated by unfolding of the cristae through alteration of the membrane diffusion barrier, such as during mitochondrial swelling (50). For this reason, we speculated that the lack of IF_1 might also favour mitochondrial Ca^{2+} accumulation by promoting basal levels of OMA1-dependent destabilization of OPA1 oligomers. The activation of the mitochondrial zinc metallopeptidase OMA1, and the subsequent proteolytic degradation of OPA1, rapidly occur under stress conditions and are prevented by IF_1 (33). To test this hypothesis,

we measured intracellular Ca^{2+} mobilization following TPG administration in IF_1 KD cells down-regulated for OMA1. Interestingly, this was sufficient to reduce mitochondrial Ca^{2+} uptake to levels comparable to control cells (**Figure 3A,B**), thus restoring the normal TPG-induced cytosolic Ca^{2+} response (**Figure 3C,D**). Moreover, knocking-down OMA1 inhibited the increase in MCU protein levels observed in IF_1 KD cells (**Figure 3E,F**), while leaving the expression of ATPB and VDAC unchanged (**Figure 3H,I**).

Although the m-AAA protease subunits SPG7 and AFG3L2 take part in the degradation of non-assembled EMRE, ensuring gatekeeping of MCU (51,52), there is no evidence to date for a direct role of OMA1 in the proteolytic regulation of the MCU complex. Nevertheless, OMA1 KO mice have been recently shown to be protected against heart failure (53). In this animal model, cristae integrity is protected (i), there is lower mtROS-mediated oxidative damage (ii) and reduced energetic collapse following isoproterenol administration (iii). Furthermore it also prevents isoproterenol-induced MCU upregulation and mitochondrial Ca^{2+} overload (53). To test whether silencing of OMA1 in IF_1 KD cells might prevent MCU upregulation by restoring mitochondrial function and promoting ATP synthesis, we repeated measurements of intracellular ATP levels after silencing of OMA1 (**Figure 3J**). Down-regulation of the peptidase significantly reduced the loss of ATP production in cells depleted of IF_1, further confirming that the activation of OMA1 critically promotes mitochondrial dysfunction in the absence of IF_1.

Discussion

With this work, we demonstrated that IF_1 is required for maintaining mitochondrial Ca^{2+} homeostasis. Our data prove that IF_1 down-regulation triggers an AMPK-mediated mitochondrial retrograde signalling response that results in the CREB-dependent over-expression of MCU, and leads to the increased propensity of mitochondria to accumulate Ca^{2+} in response to stimuli emptying the ER stores (as illustrated in **Figure 4**). While this may represent an adaptive mechanism to overcome the mitochondrial bioenergetic imbalance and oxidative stress caused by the loss of IF_1 (33,38,54), it might also contribute to the structural derangement that characterizes IF_1 KD cells (**Figure 1**) (33). Indeed, the activation of the AMPK signalling pathway following IF_1 depletion (**Figure 2**) (48) might be exploited by cells to promote their growth and metabolic reprogramming (55), thereby compensating for the drop in ATP that characterizes IF_1 KD cells (**Figure 2**) (38) through CREB-induced MCU upregulation. A higher intramitochondrial Ca^{2+} availability in IF_1 KD cells can counteract the decline in oxidative phosphorylation, but contemporaneously set the conditions for structural derangements. In accordance with this hypothesis, IF_1 KD cells show alteration in their mitochondrial structure (**Figure 1**) despite no changes in their function (**Supp. Figure 1A**). Interestingly, MCU activity and increased intramitochondrial Ca^{2+} levels are required for the constriction of the inner mitochondrial membrane (IMM) during mitochondrial fission (56). Moreover, in conditions of impaired oxidative metabolism, AMPK activation also promotes mitochondrial fragmentation via mitochondrial fission factor (Mff)-mediated Drp1 recruitment (57). Therefore, the higher mitochondrial Ca^{2+} uptake capacity of IF_1 KD cells, along with the AMPK activity, might inscrease susceptibility of these cells to undergo mitochondrial fragmentation (30).

We also found that silencing of the mitochondrial protease OMA1 restores normal MCU levels and mitochondrial Ca^{2+} uptake (**Figure 3**). This indicates that OMA1 is involved in the pathway activated by IF_1 loss. As we previously described, the over-expression of IF_1 prevents the stress-triggered activation of OMA1, thereby limiting the processing of its target OPA1 and counteracting mitochondrial cristae remodelling (33). Here we prove that reduced IF_1 levels correlate with increased MCU expression and mitochondrial Ca^{2+} buffering capacity only in the presence of OMA1, suggesting that IF_1 controls OMA1 activity even under resting conditions. We can therefore hypothesize that the alteration in the mitochondrial ultrastructure observed in IF_1-

depleted cells is caused by a combinatory effect of high Ca^{2+} and OMA1 stabilization through ATP depletion. Moreover, our data suggest that the mitochondrial ultrastructure regulates mitochondrial Ca^{2+} buffering capacity. Indeed, the higher resting-state levels of OMA1-mediated OPA1 degradation of IF_1 KD cells (33) reinforce the mitochondrial accumulation of Ca^{2+} dependent on MCU upregulation. Restoring OPA1 function through OMA1 inhibition in IF_1 KD cells would favour folding of the cristae and respiratory chain supercomplex assembly, improving both mitochondrial bioenergetics and function. This in turn reduces AMPK activation by restoring suboptimal ATP levels (**Figure 3**). Up to date, the studies on IF_1 have mainly been focused on dissecting its function in pathological scenario, such as hypoxia/ischaemia and cancer.

Collectively, our data prove that loss of the inhibitory protein impairs mitochondrial Ca^{2+} import. This might entail important consequences in both cell physiology and pathology, as it might facilitate mitochondrial Ca^{2+} overload, contributing to the derangement of mitochondrial cristae structure and swelling of the organelle. IF_1 KD cells are characterized by a substantial increase in the average cristae width, an event that, in combination with the augment in active MCU complexes, might favour Ca^{2+} entry. Nevertheless, definitive evidence confirming that cristae enlargement has a direct effect on mitochondrial Ca^{2+} dynamics is still missing. Alongside, the relationship between mitochondrial Ca^{2+} handling and structure is yet poorly understood. Our work proposes that IF_1 is a key player in this pathway, as well as a molecular mediator of the retrograde response that links impaired mitochondrial bioenergetics and structure to alterations in intracellular signalling following-deregulated Ca^{2+} buffering.

Acknowledgments

The research activities lead by M.C. are supported by the following funders, who are gratefully acknowledged: The European Research Council Consolidator Grant COG 2018 - 819600_FIRM; Biotechnology and Biological Sciences Research Council [grant numbers BB/M010384/1 and BB/N007042/1]; The Petplan Charitable Trust; LAM-Bighi Grant Initiative. FIRB-Research Grant Consolidator Grant 2 [grant number: RBFR13P392], Italian Ministry of Health [IFO14/01/R/52]. O.C. has been supported by a Fellowhsip of the Regione Lazio.

Competing Interests

There are no competing interests of any nature to report.

Legends to Figures

Figure 1: IF_1 knock-down induces mitochondrial intracristal space enlargement and enhances mitochondrial Ca^{2+} uptake in HeLa cells.

(A) Representative electron micrographs of mitochondrial ultrastructure in Scrm and IF_1 KD cells (scale bar: 500 nm).

(B) Quantification of average cristae width in the two cell lines.

(C) Prototypical traces of changes in $[Ca^{2+}]_m$, visualized with 2mtGCaMP6m, upon administration of 100 µM His in Scrm and IF_1 KD cells.

(D-E) Bar charts of average peak (D) and basal (E) $[Ca^{2+}]_m$ in the two cell lines.

(F) Representative Fura-2, AM traces describing $[Ca^{2+}]_i$ dynamics in Scrm and IF_1 KD cells following administration of 100 µM His. (G) Bar chart of average peak $[Ca^{2+}]_i$.

(H) Exemplary traces of TPG-induced mitochondrial Ca^{2+} uptake in Scrm and IF_1 KD cells transfected with 2mtGCaMP6m and challenged with 100 nM TPG.

(I) Average 2mtGCaMP6m fluorescence ratio at plateau in the two cell lines.

(J) Prototypical example of TPG-evoked $[Ca^{2+}]_i$ rise in Scrm and IF_1 KD cells loaded with Fura-2, AM and exposed to 100 nM TPG.

(K) Quantification of average Fura-2, AM plateau fluorescence ratio in the two cell lines.

Figure 2: MCU is upregulated in IF_1 KD cells via increased AMPK/CREB signalling.

(A) Representative Western blot of three independent experiments to measure the protein levels of MCU in Scrm and IF_1 KD cells. Lysates were also tested for VDAC1, ATPB and IF_1. GAPDH was used as loading control.

(B-E) Bar charts reporting quantitative analysis of the protein levels of MCU (B), IF_1 (C), VDAC1 (D) and ATPB (E) relative to GAPDH.

(F) Gene expression analysis of MCU complex subunits in IF_1 KD and Scrm cells (blue line). β-actin (ACTB) was used as internal control. (G) Averaged ratios between the mRNA levels of MCU and the other subunits of the complex.

(H) Cellular protein levels of AMPKα and phospho-AMPKα (Thr172) in Scrm and IF_1 KD cells, detected via Western blotting.

(I-K) Quantification of the protein levels of AMPKα (I), phospho-AMPKα (J) and their relative ratio (K). Vinculin was used as loading control.

(L) Western blot analysis of CREB and phospho-CREB (Ser133) levels in Scrm and IF_1 KD cells.

(M-O) Densitometry analysis of CREB (M) and phospho-CREB (N) relative to Vinculin (loading control). The ratio of expression between phophorylated and total CREB was also calculated (O).

(P) Measurement of intracellular ATP levels in Scrm and IF_1 KD cells with an ATP-based luminescence assay.

(Q) Western blot analysis of MCU levels in Scrm and IF_1 KD cells after 4 hours treatment with 10 μM forskolin. DMSO was used as vehicle. IF_1 protein band is shown to confirm efficient knock-down.

(R) Densitometry analysis of MCU protein levels relative to vinculin (loading control).

(S) qPCR analysis of MCU mRNA levels in Scrm and IF_1 KD cells after 4 hours treatment with 10 μM forskolin.

Figure 3: OMA1 knockdown rescues MCU-mediated increase in mitochondrial Ca^{2+}-uptake in IF_1 KD HeLa cells by restoring intracellular ATP levels.

(A) Prototypical traces of changes in $[Ca^{2+}]_m$ in Scrm and IF_1 KD cells co-transfected with 2mtGCaMP and either an siRNA against OMA1 (siOMA1) or a non-silencing siRNA (siNC), and treated with TPG (100 nM).

(B) Quantification of average 2mtGCaMP6m fluorescence ratio at plateau after addition of TPG in the two cell lines.

(C) Representative traces of $[Ca^{2+}]_i$ dynamics following TPG treatment in Scrm and IF_1 KD cells.

(D) Column diagram of average Fura-2, AM fluorescence ratio at plateau.

(E) Representative Western blot of MCU in Scrm and IF_1 KD cells transfected with either siNC or siOMA1; OMA1, VDAC1, ATPB and GAPDH (loading control) protein levels were also detected.

(F-I) Quantification of MCU (F), OMA1 (G), VDAC1 (H) and ATPB (I) protein expression levels relative to GAPDH.

(J) Measurements of total intracellular ATP levels in Scrm and IF_1 KD cells transfected with either siNC or OMA1 siRNA via ATP-based luminescence.

Figure 4: Proposed model for IF_1-dependent control of mitochondrial Ca^{2+} uptake.

In IF_1-depleted HeLa cells (A), the decline in cellular ATP levels induces the AMPK-mediated, CREB-dependent transcription of genes encoding the MCU complex, causing increased mitochondrial Ca^{2+} uptake following emptying of the ER stores. This situation is reverted by knocking-down OMA1 (B).

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