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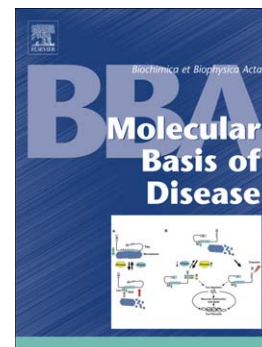
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## Calcium and ATP handling in human NADH:ubiquinone oxidoreductase deficiency

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**Running title:** Calcium and ATP handling in human complex I deficiency

**Abbreviations:** [ATP]<sub>c</sub>, cytosolic ATP concentration; [ATP]<sub>m</sub>, mitochondrial ATP concentration; CI, complex I or NADH:ubiquinone oxidoreductase; [Ca<sup>2+</sup>]<sub>c</sub>, cytosolic Ca<sup>2+</sup> concentration; [Ca<sup>2+</sup>]<sub>ER</sub>, ER Ca<sup>2+</sup> concentration; [Ca<sup>2+</sup>]<sub>m</sub>, mitochondrial Ca<sup>2+</sup> concentration; ERCa, endoplasmic reticulum Ca<sup>2+</sup> content; ETC, electron transport chain; *F*, mitochondrial formfactor; HG, hyperglycemia; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; MCU, mitochondrial Ca<sup>2+</sup> uniporter; *N<sub>c</sub>*, number of mitochondria per cell; NDUF, NADH dehydrogenase ubiquinone flavoprotein; OXPHOS, oxidative phosphorylation; PM, plasma membrane; PMCA, plasma membrane Ca<sup>2+</sup> ATPase; PMF, proton motive force; PTP, permeability transition pore; ROS, reactive oxygen species; SERCA, sarco/endoplasmic reticulum Ca<sup>2+</sup>-ATPase; VDAC, voltage-dependent anion channel.

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**ABSTRACT**

Proper cell functioning requires precise coordination between mitochondrial ATP production and local energy demand. Ionic calcium ( $\text{Ca}^{2+}$ ) plays a central role in this coupling because it activates mitochondrial oxidative phosphorylation (OXPHOS) during hormonal and electrical cell stimulation. To determine how mitochondrial dysfunction affects cytosolic and mitochondrial  $\text{Ca}^{2+}$ /ATP handling, we performed life-cell quantification of these parameters in fibroblast cell lines derived from healthy subjects and patients with isolated deficiency of the first OXPHOS complex (CI).

In resting patient cells, CI deficiency was associated with a normal mitochondrial ( $[\text{ATP}]_m$ ) and cytosolic ( $[\text{ATP}]_c$ ) ATP concentration, a normal cytosolic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_c$ ), but a reduced  $\text{Ca}^{2+}$  content of the endoplasmic reticulum (ER). Furthermore, cellular NAD(P)H levels were increased, mitochondrial membrane potential was slightly depolarized, reactive oxygen species (ROS) levels were elevated and mitochondrial shape was altered. Upon stimulation with Bradykinin (Bk), the peak increases in  $[\text{Ca}^{2+}]_c$ , mitochondrial  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_m$ ),  $[\text{ATP}]_c$  and  $[\text{ATP}]_m$  were reduced in patient cells. In agreement with these results, ATP-dependent  $\text{Ca}^{2+}$  removal from the cytosol was slower. Here, we review the interconnection between cytosolic, endoplasmic reticular and mitochondrial  $\text{Ca}^{2+}$  and ATP handling, and summarize our findings in patient fibroblasts in an integrative model.

## INTRODUCTION

Mitochondria generate ATP by the oxidative phosphorylation (OXPHOS) system located in the mitochondrial inner membrane (MIM; **Smeitink et al., 2001**). When ATP requirement increases, for instance when cell activity is triggered by a hormonal or electrical stimulus, OXPHOS-derived ATP production is increased to match cellular energy demand. A key player in this process is ionic calcium ( $\text{Ca}^{2+}$ ), which enters the cytosol from the endoplasmic/sarcoplasmic reticulum (ER/SR) and/or the extracellular medium. As a consequence, the cytosolic  $[\text{Ca}^{2+}]$  ( $[\text{Ca}^{2+}]_c$ ) is increased and  $\text{Ca}^{2+}$  is taken up by mitochondria to stimulate mitochondrial ATP production (**Rutter & Rizzuto, 2000**). The ATP generated is exported to the cytosol to fuel cytosolic ATP-demanding processes like  $\text{Ca}^{2+}$  uptake in the ER by the sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) and/or  $\text{Ca}^{2+}$  extrusion across the plasma membrane (PM) by PM  $\text{Ca}^{2+}$ -ATPases (PMCA). As a consequence,  $[\text{Ca}^{2+}]_c$  rapidly returns to basal levels again (**Frieden et al., 2005; Spat et al., 2008; Willems et al., 2008**). In this review we discuss the interconnection between cytosolic, endoplasmic reticular and mitochondrial  $\text{Ca}^{2+}$  and ATP handling (**section 1**) and how mitochondrial CI deficiency may affect this coupling (**section 2**).

### 1. Interconnection between cellular $\text{Ca}^{2+}$ and ATP handling

#### 1.1. Hormone-induced $\text{Ca}^{2+}$ release from the endoplasmic reticulum

The ER not only plays a key role in lipid and protein synthesis, folding and post-translational modification but also constitutes the main intracellular  $\text{Ca}^{2+}$  storage organelle (**Baumann & Walz, 2001; Pizzo & Pozzan, 2007**). Inside the lumen of the ER, part of the  $\text{Ca}^{2+}$  is free, whereas the remainder is bound to  $\text{Ca}^{2+}$ -binding proteins (**Gelebart et al., 2005**). Estimation of the free luminal  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_{\text{ER}}$ ) yielded values of 0.1-0.8 mM under resting conditions (**Spat et al., 2008**). One of the main ER  $\text{Ca}^{2+}$ -binding proteins, calreticulin, is also involved in quality control and folding of newly-synthesized (glyco)proteins (**Trombetta & Parodi, 2003**). This suggests that these processes are under regulatory control of  $[\text{Ca}^{2+}]_{\text{ER}}$  (**Berridge, 2002; Burdakov et al., 2005; Michalak et al., 2002; Szabo et al., 2008; Grolach et al., 2006**).

At any point in time, the total  $\text{Ca}^{2+}$  content of the ER (ERCa) depends on the amount of ER  $\text{Ca}^{2+}$  binding proteins and the balance between passive  $\text{Ca}^{2+}$  leak,  $\text{Ca}^{2+}$  release via specific  $\text{Ca}^{2+}$  channels such as the inositol 1,4,5 trisphosphate ( $\text{InsP}_3$ ) receptor ( $\text{InsP}_3\text{R}$ ) and the activity of the ER  $\text{Ca}^{2+}$ -pumps (SERCAs). Blocking of SERCA pumps in the absence of extracellular  $\text{Ca}^{2+}$ , induces an instantaneous increase in  $[\text{Ca}^{2+}]_c$  in resting human skin fibroblasts (**Visch et al., 2004**), indicating that maintenance of ERCa requires continuous SERCA action to compensate for passive  $\text{Ca}^{2+}$  leak. It is likely that the ATP required to fuel the SERCAs is generated by mitochondria operating in close vicinity of the ER

(Rutter & Rizzuto, 2000; Pizzo & Pozzan, 2007; Spat et al., 2008; Willems et al., 2008). In this way, cells also maintain a low resting  $[Ca^{2+}]_c$ , which is not only a prerequisite for  $Ca^{2+}$  signaling, but also prevents possible toxic effects of  $Ca^{2+}$  like ATP precipitation, DNA breakdown and activation of  $Ca^{2+}$ -dependent proteases (Matson, 2007).

Many hormones trigger the production of  $InsP_3$  via binding to a heterotrimeric G protein-activating receptor at the PM (Fig. 1B). The GTP-bound  $\alpha$ -subunit of the G protein then stimulates the action of PM-bound phospholipase C beta (PLC- $\beta$ ), which cleaves phosphatidylinositol 4,5-bisphosphate ( $PtdInsP_2$ ) into 1,2-diacylglycerol (DAG), an activator of protein kinase C (PKC), and  $InsP_3$ . The latter molecule diffuses into the cytosol to stimulate the  $InsP_3R$ -mediated release of  $Ca^{2+}$  from the ER into the cytosol. As a result,  $[Ca^{2+}]_c$  increases and, in healthy cells, is rapidly returned to pre-stimulus levels by mainly SERCA and/or PMCA action. In many cell types, hormone-induced cell activation is mediated by periodic rises in, often local,  $[Ca^{2+}]_c$  to avoid the toxic side effects of  $Ca^{2+}$  (see above) and, more importantly, to allow high-fidelity signal transduction (Goldbeter et al., 1990; Dupont et al., 2003). Thus, this oscillatory signaling mode, as it is generally referred to, enables coding of the signaling information in the frequency, amplitude and/or shape of the  $[Ca^{2+}]_c$  rises and is, in general, only observed when low (physiological) concentrations of hormone are used (Torres et al., 2001; Berridge, 2002; Koopman et al., 2003; Koopman et al., 2005a). The  $InsP_3R$ , exists as homo and/or heterotetramers (Mikoshiba, 2007). In mammals, three  $InsP_3R$  isoforms have been identified ( $InsP_3R_1$ ,  $InsP_3R_2$ ,  $InsP_3R_3$ ) that are regulated by  $[Ca^{2+}]_c$ ,  $[Ca^{2+}]_{ER}$ , protein kinases and specific binding proteins (Foskett, 2007). The latter include homer, protein 4.1N, huntingtin-associated protein-1A, protein phosphatases (PPI and PP2A), RACK1, ankyrin, chromogranin, carbonic anhydrase-related protein, IRBIT,  $Na^+/K^+$ -ATPase, and ERp44. This suggests that  $InsP_3Rs$  form macro signal complexes that function as centers of signaling cascades (Mikoshiba, 2007).

## 1.2. The ER and mitochondria are in close vicinity

Evidence has been presented that at least part of the ER is in close vicinity of mitochondria (Fig. 1A). This juxtaposition creates an ER-mitochondrial ‘synapse’ that is thought to allow efficient exchange of  $Ca^{2+}$  and metabolites (Alonso et al., 2006; Collins et al., 2001; Csordás et al., 2006; Filippin et al., 2003; Maack and O’Rourke, 2007; Pizzo and Pozzan, 2007; Rizzuto et al., 1998; Simmen et al., 2005; Szabadkai et al., 2006; Franzini-Armstrong, 2007). However, there is also evidence arguing against mitochondria–ER contacts being a general  $Ca^{2+}$  shunting mechanism (Rutter, 2006). For instance, artificial relocation of mitochondria to the perinuclear region, which is rich in ER, does not affect mitochondrial  $Ca^{2+}$  uptake in response to stimulated  $Ca^{2+}$  release from internal stores. Additionally, mitochondria are highly mobile in cells, suggesting that: (i) ER-mitochondrial contacts

are either formed between immobile parts of the ER and immobile mitochondria, (ii) mitochondria-connected parts of the ER move in parallel with mitochondria, or (iii) ER-mitochondrial contacts only exist transiently (**Rutter, 2006**). Furthermore, mitochondria can exist as small bean-shaped organelles and as more elongated tubular structures. Moreover, they can form intricate (sub)reticula (**Twig et al., 2006; Koopman et al., 2007; Benard et al., 2008**). Therefore, nature, functionality and amount of ER-mitochondrial contacts most probably depend on the type of cell and/or its metabolic condition. Taken together, it appears that a close proximity between ER and mitochondria alone is not sufficient for rapid  $\text{Ca}^{2+}$  transfer between the both organelles and that the latter requires assembly of an appropriate molecular machinery (**Rutter, 2006**). In this respect, recent evidence suggested that the ER-mitochondrial synapse is maintained by 10-25 nm tethers, physically connecting both organelles (**Csordás et al., 2006**). These tethers were shown to consist of the mitochondrial outer membrane (MOM)-protein VDAC1 (voltage-dependent anion channel 1 or porin), the chaperone glucose-related protein 75 (grp75 or mitochondrial heat shock protein 70; mtHSP70), and the  $\text{InsP}_3\text{R}$  (**Szabadkai et al., 2006**). In agreement with its putative role in ER-mitochondria tethering, overexpression of VDAC in HeLa cells and skeletal myotubes enhanced the amplitude of the agonist-induced rise in  $[\text{Ca}^{2+}]_m$  and shortened the delay between the increase in  $[\text{Ca}^{2+}]_c$  and  $[\text{Ca}^{2+}]_m$  (**Rapizzi et al., 2002**). Functionally, VDAC-overexpressing cells were more susceptible to ceramide-induced cell death, suggesting that increased mitochondrial  $\text{Ca}^{2+}$  uptake stimulates the process of apoptosis. Similarly, overexpression of a grp75 mutant lacking the mitochondrial presequence also enhanced mitochondrial  $\text{Ca}^{2+}$  accumulation, implying that this protein plays a role in  $\text{Ca}^{2+}$  handling independent from its chaperone activity in the mitochondrial matrix (**Szabadkai et al., 2006**). Other recent evidence suggested that the ER protein sigma-1 receptor (Sig-1R), implicated in neuroprotection, carcinogenesis, and neuroplasticity and found to be located at the mitochondria-associated ER membrane (MAM), senses  $[\text{Ca}^{2+}]_{\text{ER}}$  and might thus be involved in the regulation of ER-mitochondria  $\text{Ca}^{2+}$  signals (**Hayashi et al., 2007**). Finally, very recent evidence implicated mitofusin 2, a mitochondrial dynamin-related protein demonstrated to be enriched at the ER-mitochondria interface, in the regulation of mitochondrial  $\text{Ca}^{2+}$  uptake. It was found that its down-regulation disturbed ER-mitochondria interactions leading to a decreased mitochondrial  $\text{Ca}^{2+}$  uptake in response to  $\text{InsP}_3$  generating stimuli (**Martins de Brito and Scorrano, 2008**). It appears that the distance between ER and mitochondrion, crucial for ER-mitochondrial  $\text{Ca}^{2+}$  transmission, is (co)regulated by  $[\text{Ca}^{2+}]_c$  and determines the susceptibility to mitochondrial  $\text{Ca}^{2+}$  overload and opening of the mitochondrial permeability transition pore (**Csordás et al., 2006**). Free (local)  $[\text{Ca}^{2+}]_c < 100$  nM favors ER-mitochondria dissociation, whereas  $[\text{Ca}^{2+}]_c > 1$   $\mu\text{M}$  favors close association between these organelles (**Wang et al., 2000**). This suggests that ER subdomains are less

closely associated with mitochondria in resting cells, whereas association occurs during cell stimulation.

### 1.3. Mitochondrial $\text{Ca}^{2+}$ uptake, release and ATP generation

During cell stimulation, cytosolic  $\text{Ca}^{2+}$  rapidly enters the mitochondrial matrix by action of the  $\Delta\psi$ -dependent mitochondrial  $\text{Ca}^{2+}$  uniporter (MCU) leading to an increase in  $[\text{Ca}^{2+}]_m$  (Rutter & Rizzuto, 2000; Pizzo & Pozzan, 2007). The MCU is half-maximally activated at a  $[\text{Ca}^{2+}]_c$  of  $\sim 10$ - $20 \mu\text{M}$  and has a maximal  $\text{Ca}^{2+}$  flux of  $\sim 2 \cdot 10^4 \text{ Ca}^{2+} \cdot \text{s}^{-1}$  per MCU molecule (Maack & O'Rourke, 2007). The close ER-mitochondrial juxtaposition allows the  $[\text{Ca}^{2+}]_c$  within the  $\text{Ca}^{2+}$  microdomain (Fig. 1B) to become sufficiently high for MCU activation. Using confocal imaging of Rhod-2-loaded HeLa cells, Bootman and colleagues demonstrated that mitochondria will accumulate  $\text{Ca}^{2+}$  regardless whether it is released from the ER by  $\text{InsP}_3$ , enters across the PM or leaks from the ER (Collins et al., 2001). However, in agreement with the  $\text{InsP}_3\text{R}$  being involved in ER-mitochondrial tethering (see above), the rate of mitochondrial  $\text{Ca}^{2+}$  uptake was greatest for  $\text{InsP}_3$ -evoked  $\text{Ca}^{2+}$  signals.

Interestingly, when glomerular afferent arteriolar smooth muscle cells (PGASMC) were chronically pre-treated with Transforming Growth Factor- $\beta$  (TGF- $\beta$ ), subsequent hormone-induced  $[\text{Ca}^{2+}]_c$  and  $[\text{Ca}^{2+}]_m$  rises were decreased (Pacher et al., 2008). Although TGF- $\beta$  down-regulated both  $\text{InsP}_3\text{R}_1$  and  $\text{InsP}_3\text{R}_3$ , ER  $\text{Ca}^{2+}$  storage, mitochondrial distribution and ER-mitochondrial contacts were not affected. It was concluded that TGF- $\beta$  causes uncoupling of mitochondria from ER  $\text{Ca}^{2+}$  release by decreasing the  $\text{InsP}_3\text{R}$ -mediated  $\text{Ca}^{2+}$  efflux, thus hampering the build-up of the  $[\text{Ca}^{2+}]_c$  within the ER-mitochondrial  $\text{Ca}^{2+}$  microdomain.

The decay phase of  $[\text{Ca}^{2+}]_m$  is much slower than the MCU-mediated rising phase, and is primarily mediated by  $\text{Na}^+/\text{Ca}^{2+}$  (NCX) exchange across the MIM. The NCX is thought to be electrogenic because it exchanges 1  $\text{Ca}^{2+}$  for 3  $\text{Na}^+$  with a  $K_m$  for  $\text{Na}^+$  of 8 mM (Maack & O'Rourke, 2007). MCU stimulation by 4,4',4''-(4-propyl-[ $^1\text{H}$ ]-pyrazole-1,3,5-triyl)trisphenol (PPT) or kaempferol increased the histamine-induced ER  $\text{Ca}^{2+}$  release in HeLa cells and fibroblasts (Vay et al., 2007). This effect was enhanced by simultaneous inhibition of NCX with the benzothiazepine CGP37157, suggesting that mitochondrial  $\text{Ca}^{2+}$  uptake and release control feedback inhibition of  $\text{InsP}_3\text{Rs}$  within the ER-mitochondrial  $\text{Ca}^{2+}$  microdomain (Fig. 1B). Interestingly, PPT and kaempferol induced  $[\text{Ca}^{2+}]_c$  oscillations in previously silent fibroblasts. This suggests that also in the absence of hormone stimulation, the occurrence of oscillatory  $\text{Ca}^{2+}$  signals depends on the rates of mitochondrial  $\text{Ca}^{2+}$  uptake and release, which modulate  $\text{InsP}_3\text{R}$  activation. In HeLa cells, inhibition of mitochondrial NCX by CGP37157 during histamine stimulation changed the pattern of histamine-induced  $[\text{Ca}^{2+}]_c$  oscillations (Hernandez-SanMiguel et al., 2006). Similarly, CGP37157 increased the frequency of

$[Ca^{2+}]_c$  oscillations in human fibroblasts with spontaneous activity and induced the generation of oscillations in cells without spontaneous activity. These findings suggest that mitochondrial NCX directly modulates  $InsP_3$ -induced  $Ca^{2+}$  release and thereby controls  $[Ca^{2+}]_c$  oscillations.

Within the mitochondrial matrix, an elevation in  $[Ca^{2+}]_m$  stimulates respiration and increases mitochondrial  $[ATP]$  ( $[ATP]_m$ ) (Jouaville et al., 1999; Maack & O'Rourke, 2007; Visch et al., 2004; Visch et al., 2006a; Willems et al., 2008). Mitochondrial ATP production is carried out by the OXPHOS system, which consists of five MIM-embedded multi-protein complexes (Smeitink et al., 2001). The system is primarily fueled by NADH and  $FADH_2$ , generated by the tricarboxylic acid (TCA) cycle. Electrons are abstracted from NADH and  $FADH_2$  at complex I (CI or NADH:ubiquinone oxidoreductase; EC 1.6.5.3) and II (CII or succinate dehydrogenase; EC 1.3.5.1), respectively, and donated to complex III (CIII or ubiquinol cytochrome c reductase; EC 1.10.2.2) via the MIM-bound electron transporter ubiquinone. Next, cytochrome *c* transports the electrons to complex IV (CIV or cytochrome *c* oxidase; EC 1.9.3.1), where they are donated to molecular oxygen ( $O_2$ ) leading to the formation of water ( $H_2O$ ). At CI, CIII and CIV protons are translocated across the MIM to create a proton-motive force (PMF) that consists of a chemical ( $\Delta pH$ ) and electrical component ( $\Delta \psi$ ). This PMF is utilized at complex V (CV or ATP synthase; EC 3.6.3.14) to form ATP from ADP and  $P_i$ .

Three key dehydrogenases of the TCA cycle (pyruvate dehydrogenase, isocitrate dehydrogenase,  $\alpha$ -ketoglutarate dehydrogenase), as well as CV, are activated by  $Ca^{2+}$  (Rutter & Rizzuto, 2000; Territo et al., 2000). Computational analysis also predicts that multi-site OXPHOS activation is required for efficient matching of mitochondrial ATP production with cellular demand (Korzeniewski, 2007). The  $K_a$  for activation of the TCA cycle dehydrogenases is within the range of 0.7-1  $\mu M$  (Maack & O'Rourke, 2007), compatible with the observed increases in  $[Ca^{2+}]_m$ , (Brini et al., 1999; Visch et al., 2004).

Once produced, mitochondrial ATP is transported out of the mitochondrion by the adenine nucleotide translocase (ANT; Klingenberg et al., 2008). This transporter exchanges ATP against ADP across the MIM and has also been proposed to be involved in mitochondrial  $Ca^{2+}$  homeostasis. The latter is illustrated by the fact that ANT overexpression reduced the histamine-induced peak increase in  $[Ca^{2+}]_m$  in HeLa cells (Wieckowski et al., 2006). This effect was paralleled by alterations in mitochondrial shape and also reduced ER $Ca$ , resulting in a small decrease in peak  $[Ca^{2+}]_c$  during histamine stimulation.

Within the cytosol, the ATP generated is used to fuel, among others, the SERCA and/or PMCA (Fig. 2B), which return  $[Ca^{2+}]_c$  to the pre-stimulus level (Pizzo & Pozzan, 2007). The  $[Ca^{2+}]_c$  in close vicinity of the SERCA/PMCA also affects the action of this pump. In this way, mitochondrial ATP

production and  $\text{Ca}^{2+}$  uptake may exert local control on PMCA action and SERCA activity in the adjacent ER (Spat et al., 2008).

## 2. ER, cytosolic and mitochondrial $\text{Ca}^{2+}$ and ATP handling in isolated complex I deficiency

### 2.1. Isolated complex I deficiency

CI is the largest OXPHOS complex (~1 MDa) and constitutes the entry point of electrons in the electron transport chain (ETC; Smeitink et al., 2001). Structurally, CI is L-shaped and consists of 45 different subunits, 14 of which are essential for catalytic function (Brandt, 2006). These 'core' subunits are encoded by genes on both the mitochondrial (mtDNA) and nuclear DNA (nDNA). The seven mtDNA-encoded core subunits (ND1, ND2, ND3, ND4, ND4L, ND5 and ND6) all are part of the membrane arm of CI, whereas the nDNA-encoded ones (NDUFV1, NDUFV2, NDUFS1, NDUFS2, NDUFS3, NDUFS7 and NDUFS8) are mainly localized in the matrix-protruding arm of the complex. The remaining 31 subunits are all nDNA-encoded and their function is largely unknown. *In vivo* assembly of functional CI is thought to occur by stepwise combination of pre-assembled modules at the MIM (Dieteren et al., 2008) and requires assistance of assembly chaperones, seven of which have currently been identified (CIA30/NDUFAF1, Mimitin/B17.2L, NDUFA12L, C6orf66, C8orf38, C20orf7 and Ecsit; Dunning et al., 2007; Ogilvie et al., 2005; Pagliarini et al., 2008; Lazarou et al., 2008; Sugiana et al., 2008; Vogel et al., 2007a; Vogel et al., 2007b).

Deficiency of CI (OMIM 252010) was first described in humans 30 years ago (Morgan-Hughes et al., 1979). A reduced enzyme activity of this complex, for diagnostic purposes usually determined in skeletal muscle and cultured skin fibroblasts, leads to multi-system disorders affecting predominantly organs and tissues with a high-energy demand like brain, heart, and skeletal muscle (Smeitink et al., 2004). Although patients with isolated CI deficiency due to nDNA mutations may present with a variety of signs and symptoms, Leigh disease is the most frequent, accounting for almost half of the cases (Janssen et al., 2006; Lazarou et al., 2008). Leigh disease is an early-onset (mostly during the first year of life) fatal neurodegenerative disorder that is typically characterized by symmetrical lesions of necrosis and capillary proliferation in variable regions of the central nervous system. Clinical signs and symptoms include optic atrophy, ophthalmoparesis, muscular hypotonia, ataxia and dystonia. In all cases the disease is fatal (most children do not reach the age of one year).

Currently, mutations in 12 different nDNA-encoded CI subunits have been linked to isolated CI deficiency (NDUFS1, NDUFS2, NDUFS4, NDUFS6, NDUFS7, NDUFS8, NDUFV1, NDUFV2, NDUFA1, NDUFA2, NDUFA8, NDUFA11; Fernandez-Moreira et al., 2007; Berger et al., 2008; Hoefs et al., 2008; Janssen et al., 2006; Lazarou et al., 2008). Additionally, pathological mutations

in six complex I assembly factors (CIA30/NDUFAF1; Mimitin/B17.2L, NDUFA12L, C6orf66, C8orf38 and C20orf7) have been reported (Barghuti et al., 2008; Dunning et al., 2007; Ogilvie et al., 2005; Lazarou et al., 2008; Pagliarini et al., 2008; Saada et al., 2008; Sugiana et al., 2008; Vogel et al., 2007b). Currently, effective treatment strategies for isolated CI deficiency are lacking due to the limited insight into its cytopathology. Therefore, in the past years, we systematically investigated the possible consequences of nDNA-encoded CI mutations in patient fibroblasts. In doing so, we found aberrations in  $\text{Ca}^{2+}$  and ATP homeostasis, ROS handling and mitochondrial morphology (summarized in: Koopman et al., 2007; Willems et al., 2008).

## 2.2. Mitochondrial $\text{Ca}^{2+}$ and ATP handling in patient skin fibroblasts

Our previous studies with a cohort of skin fibroblast lines from patients with isolated CI deficiency revealed that CI mutations primarily lead to a reduced amount of fully assembled and catalytically active CI by decreasing the rate of assembly and/or disturbing the stability of the holo-complex (Ugalde et al., 2004; Verkaart et al., 2007a; Koopman et al., 2008b).

Quantification of ERCa in resting cells, revealed that the ER contained less  $\text{Ca}^{2+}$  in patient cells, which displayed a reduced peak increase in  $[\text{Ca}^{2+}]_c$ ,  $[\text{Ca}^{2+}]_m$  and  $[\text{ATP}]_m$  upon stimulation with the  $\text{InsP}_3$ -generating hormone bradykinin (Bk; see below and: Visch et al., 2004; Visch et al., 2006a; Willems et al., 2008). This suggests that  $[\text{ATP}]_c$  might be too low in resting patient cells to allow sufficient ATP fueling of the SERCAs. To investigate this possibility we developed a protocol for quantification of  $[\text{ATP}]_c$  and  $[\text{ATP}]_m$ , based on a previously described method for endothelial cells (supplement of Trenker et al., 2007). Briefly, the bioluminescent ATP sensor luciferase (LUC) was expressed in the cytosol (cytLUC) or mitochondrial matrix (mitLUC; using the COX8 mitochondrial targeting sequence) of skin fibroblasts from a healthy subject and 3 patients harboring a mutation in one of the nDNA-encoded CI genes (*NDUFS4*, *NDUFS7*, *NDUSF8*; table 1). MitLUC and cytLUC were introduced into the cells using the baculoviral system (Condreay & Kost, 2007), made suitable for protein expression in human skin fibroblasts (Visch et al., 2004). Luciferase catalyzes the luminescent reaction of luciferin with ATP and oxygen. Figure 2A shows that upon addition of luciferine the bioluminescence signal gradually increased to a steady level, reflecting the resting ATP concentration. Subsequent permeabilization by digitonin in the presence of a saturating concentration of ATP yielded the maximal signal which was used for normalization of the resting signal. As depicted in figure 2B, the resting values of  $[\text{ATP}]_c$  and  $[\text{ATP}]_m$  did not significantly differ between healthy and patient fibroblasts. Although the resting values of  $[\text{ATP}]_m$  and  $[\text{ATP}]_c$  were not altered in CI-deficient patient fibroblasts, it cannot be excluded that local decreases in  $[\text{ATP}]_c$ , in the ER-mitochondrial  $\text{Ca}^{2+}$  microdomain, occur and are responsible for the observed reduction in resting ERCa (Fig. 1B).

Concerning the  $\text{Ca}^{2+}$  buffering capacity in the ER lumen, Western blot analysis of whole-cell homogenates showed that the calreticulin protein amount was increased rather than decreased in typical patient fibroblasts. Because calreticulin is the predominant  $\text{Ca}^{2+}$  binding protein of the ER, this finding disfavors the idea that the reduction in resting ERCa is due to a decrease in ER  $\text{Ca}^{2+}$  buffering capacity (**Willems et al., 2008**). Measurement of ER  $\text{Ca}^{2+}$  uptake under saturating [ATP], revealed that the maximum SERCA-pump capacity was similar in control and patient fibroblasts. Additionally, the amount of SERCA2b protein, was not altered in typical patient cells (**Willems et al., 2008**), further supporting the conclusion that ERCa is decreased because of reduced SERCA fuelling by mitochondrial ATP.

Typically, when patient cells were stimulated with Bk, transient increases in  $[\text{Ca}^{2+}]_c$ ,  $[\text{Ca}^{2+}]_m$  and  $[\text{ATP}]_m$  were observed, which peaked at a lower values than in healthy control cells (**Visch et al., 2004; Visch et al., 2006a; Willems et al., 2008**). Regression analysis of the patient data, revealed that the peak values of  $[\text{Ca}^{2+}]_c$ ,  $[\text{Ca}^{2+}]_m$  and  $[\text{ATP}]_m$  were linearly correlated, suggesting that the magnitude of the  $[\text{Ca}^{2+}]_c$  transient is the primary determinant of the  $[\text{Ca}^{2+}]_m$ -induced increase in  $[\text{ATP}]_m$ . This is compatible with a mechanism in which  $[\text{Ca}^{2+}]_m$  stimulates mitochondrial ATP production (**Jouaville et al., 1999**). Given the fact that mitochondrial  $\text{Ca}^{2+}$  uptake is  $\Delta\psi$ -dependent (**Rutter & Rizzuto, 2000; Visch et al., 2004; Valero et al., 2008**), the linear relationship between peak  $[\text{Ca}^{2+}]_c$  and peak  $[\text{Ca}^{2+}]_m$  furthermore suggests that the small  $\Delta\psi$  depolarization observed in patient fibroblasts does not significantly contribute to the observed alterations in Bk-stimulated mitochondrial  $\text{Ca}^{2+}$  and ATP handling. Quantification of the decay rate of the  $[\text{Ca}^{2+}]_c$  transient revealed a halftime ( $t_{1/2}$ ) that was inversely proportional to the peak  $[\text{ATP}]_m$ , suggesting that mitochondria-generated ATP serves to fuel SERCA-mediated  $\text{Ca}^{2+}$  reuptake by the ER (**Visch et al., 2006a; Visch et al., 2006b; Willems et al., 2008**).

Recent studies using isolated cardiomyocytes of mice with tissue-specific knockout of mitochondrial transcription factor A (Tfam), revealed that action potential-mediated  $[\text{Ca}^{2+}]_c$  transients, measured with the fluorescent indicator Fluo-3, were smaller and faster than control (**Tavi et al., 2008**). Moreover, both the  $\text{Ca}^{2+}$  content of the sarcoplasmic reticulum and the expression level of the  $\text{Ca}^{2+}$ -binding protein calsequestrin-2 were found to be reduced in Tfam knockout hearts. These results indicate that although the consequences of Tfam ablation for the stimulus-induced  $[\text{Ca}^{2+}]_c$  increase in mice cardiomyocytes resemble those in CI-deficient human skin fibroblasts, the underlying mechanism is different.

### 2.3. Cell biological consequences of complex I deficiency: an integrative model

In addition to  $\text{Ca}^{2+}$  and ATP handling, we also have investigated the consequences of isolated CI deficiency on CI assembly, OXPHOS protein expression, mitochondrial membrane potential ( $\Delta\psi$ ), reactive oxygen species (ROS) levels, thiol redox status, lipid peroxidation, NAD(P)H level, mitochondrial morphology, motility and intra-matrix protein diffusion (**Visch et al., 2004; Verkaart et al., 2007a; Koopman et al., 2005b; Koopman et al., 2008a; Koopman et al., 2008b; Willems et al., 2008**).

In patient cells (see **table 1** for typical examples), reduced expression of the CI holo-enzyme was paralleled by a (slightly) depolarized  $\Delta\psi$ , and increased ROS and NAD(P)H levels. Because the reduction in CI levels was inversely proportional to cellular ROS levels, it appears that decreasing numbers of active complexes generate increasing amounts of ROS not because of the presence of a mutated subunit but as a consequence of a decrease in cellular CI activity. The alternative explanation that increasing numbers of partially assembled complexes are responsible for the observed increase in ROS production is not supported by our finding that chronic rotenone treatment increased rather than decreased the amount of fully assembled CI (**Verkaart et al., 2007a**).

In a recent study, we investigated the effect of chronic treatment with Trolox, a water-soluble derivative of vitamin E, on cellular ROS levels and expression and activity of fully assembled CI (**Koopman et al., 2008b**). It was found that ROS levels were dramatically reduced and CI expression and activity were variably increased in healthy and patient fibroblasts. This suggests that the amount of active CI is under regulatory control of the cell's oxidative balance. By ratioing the Trolox-induced increase in CI activity and CI amount, we determined whether newly generated CI holo-complexes were catalytically active. The results obtained revealed that apart from the amount also the intrinsic activity of the complex can be significantly decreased in nDNA-inherited isolated CI deficiency. The finding that Trolox treatment increased the amount of CI might provide an experimental basis for the use of antioxidants to mitigate the deficiency. However, it is to be expected that such a treatment is only beneficial to patients with a predominant expression rather than intrinsic catalytic defect of the complex.

As discussed above (**section 2.2**), the CI deficiency-induced mitochondrial dysfunction leads to a reduced ERCa in resting cells, most probably by reduced ATP fueling of SERCAs. Alternatively, ERCa in patient cells might be reduced by functional impairment of the SERCAs and/or  $\text{InsP}_3$  receptor by increased ROS levels (**Squier, 2000; Waring, 2005; Bigelow et al., 2005; Hool & Corry, 2007**). However, maximal SERCA-pump capacity in patient fibroblasts was normal (**section 2.2**) and ERCa displayed a strong linear correlation with the Bk-induced  $\text{InsP}_3$ R-mediated peak increase in  $[\text{Ca}^{2+}]_c$ , disfavoring a role for  $\text{InsP}_3$ R malfunction (**Visch et al., 2006a**). Given the established signaling role of

$\text{Ca}^{2+}$  in the ER lumen, the reduced ER $\text{Ca}^{2+}$  might affect intra-ER and/or cytosolic signal transduction pathways. In this respect, it has been proposed that intra-ER  $\text{Ca}^{2+}$  not only functionally regulates  $\text{Ca}^{2+}$ -binding chaperones responsible for intra-ER protein folding, but also expression of these chaperones once released from the ER by cell stimulation (**Groenendyk & Michalak, 2005**). Similarly, ER $\text{Ca}^{2+}$  might regulate proteins involved in ER stress responses like the UPR (unfolded protein response) and ERAD (ER-associated degradation). To determine if such mechanisms are operational in CI-deficient patient fibroblasts, further investigations are needed.

It is likely that the elevated ROS/NADH(P)H triggers activation of adaptive responses (**Droge, 2002; Trachootham et al., 2008**). This might be related to the observed changes in mitochondrial shape, motility, intra-matrix protein diffusion and mtHSP70 expression in patient cells (**Koopman et al., 2007; Koopman et al., 2008a; Benard et al., 2008**). We previously argued that these alterations reflect a switch to a (more) glycolytic mode of ATP generation in patient fibroblasts (**Koopman et al., 2008a**). Similarly, in 143B osteosarcoma cybrid cells harboring pathogenic mtDNA point mutations in tRNA<sup>Leu</sup> the major OXPHOS defect was efficiently compensated by increased anaerobic glycolysis, so that the total ATP production rate was preserved (**Von Kleist-Retzow et al., 2007**). The latter is compatible with the normal resting  $[\text{ATP}]_m$  and  $[\text{ATP}]_c$  observed in our patient cells (**section 2.2**). The 143B cybrid cells displayed an increased ROS production, which was not paralleled by induction of antioxidant defense systems or substantial oxidative damage. Comparison of a cohort of 10 healthy and 10 CI-deficient patient cell lines also revealed no detectable differences in thiol redox status, glutathione/glutathione disulfide content, or extent of lipid peroxidation (**Verkaart et al., 2007b**). This indicates that fibroblasts of patients with isolated CI deficiency maintain their thiol redox status despite the marked increase in ROS production. Similar to our patient cells,  $\Delta\psi$  was depolarized in 143B cybrid cells and this was associated with a disturbed mitochondrial  $\text{Ca}^{2+}$  homeostasis (**Von Kleist-Retzow et al., 2007**).

In summary, the data presented in this review highlight that mutations in nDNA-encoded CI subunits have 4 major consequences: **(i)** they reduce the amount and activity of fully assembled and active CI, **(ii)** they increase ROS levels, **(iii)** they cause aberrations in  $\text{Ca}^{2+}$ /ATP handling and, **(iv)** they trigger an adaptive response. Currently, strategies targeting these consequences in order to mitigate CI deficiency are under way in our laboratory.

## FIGURE LEGENDS

**Figure 1: Interconnection between ER, cytosolic and mitochondrial  $\text{Ca}^{2+}$  and ATP handling.** (A) Contrast-optimized electron microscopy image of mitochondria in a typical healthy human skin fibroblast (CT-5120) showing the close proximity of a mitochondrion and parts of the rough ER (RER; inset). The scale bar indicates 1  $\mu\text{m}$  (for details see: **Koopman et al., 2008**). (B) Cartoon depicting the interconnection between  $\text{Ca}^{2+}$  and ATP homeostasis in the cytosol, ER and mitochondria during hormone-stimulation (see text for details). **Abbreviations:** ANT, adenine nucleotide translocase;  $[\text{ATP}]_c$ , cytosolic ATP concentration;  $[\text{ATP}]_m$ , mitochondrial ATP concentration; BkR, bradykinin-receptor; CI, complex I; CII, complex II; CIII, complex III; CIV, complex IV; CV, complex V;  $[\text{Ca}^{2+}]_c$ , cytosolic  $\text{Ca}^{2+}$  concentration;  $[\text{Ca}^{2+}]_{\text{ER}}$ , endoplasmic reticulum  $\text{Ca}^{2+}$  concentration;  $[\text{Ca}^{2+}]_m$ , mitochondrial  $\text{Ca}^{2+}$  concentration; DAG, diacylglycerol; G, G-protein;  $\text{InsP}_3$ , inositol 1,4,5-trisphosphate;  $\text{InsP}_3\text{R}$ , inositol 1,4,5-trisphosphate receptor; MIM, mitochondrial inner membrane; MOM, mitochondrial outer membrane; PDHs, pyruvate dehydrogenases;  $\text{PIP}_2$ , phosphatidylinositol 4,5-bisphosphate; PM, plasmamembrane; PMCA, plasmamembrane  $\text{Ca}^{2+}$ -ATPase;  $\text{PLC}\beta$ , phospholipase C- $\beta$ ; SERCA, sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase.

**Figure 2: Resting mitochondrial and cytosolic [ATP] in CI-deficient patient fibroblasts.** (A) Calibration of average luciferase (LUC) bioluminescence signals in the mitochondrial matrix (mitLUC; closed symbols) and cytosol (cytLUC; open symbols) in a population (25,000 cells) of healthy human skin fibroblast (CT-5120). Following application of the LUC co-factor luciferin, a steady state level of bioluminescence was reached. This signal was expressed as percentage of the maximal bioluminescence signal obtained after permeabilization of the cells with digitonin in the presence of a saturating [ATP] (10 mM). This approach allowed comparison (see inset) of the resting [ATP] in the mitochondrion and cytosol. Bioluminescence signals were recorded with a photomultiplier system as described previously (**Visch et al., 2004**). (B) Comparison of the resting [ATP] in the mitochondrial matrix (closed bars) and cytosol (open bars) in control cells (CT) and 3 different patient cell lines. Asterisks indicate significant differences with the indicated columns (\* $p < 0.05$ ; \*\* $p < 0.01$ ). More details are given in the text and **Table 1**.

**Figure 3: Cell biological consequences of isolated CI deficiency in patient fibroblasts.** Typical aberrations observed in fibroblasts of patients with isolated CI deficiency obtained by quantitative life-cell microscopy and bioluminometry (see text for details).

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**Table 1: Characteristics of typical complex I-deficient fibroblast cell lines.**

Parameter	CT-5120	P-4827	P-5175	P-6603
<b>Subunit</b>	n.a.	NDUFS4	NDUFS7	NDUFS8
<b>Subunit features</b>	n.a.	Accessory	Core	Core
<b>Mutation</b>	n.a.	R106X	V122M	R94C
<b>CI activity</b>	113	<b>58</b>	<b>68</b>	<b>18</b>
<b>PN</b>	17	7*	15*	15*
<b>Clinical phenotype</b>	n.a.	L/LL	L/LL	L/LL
<i>RESTING CELL</i>				
% $\Delta\Psi$	100 $\pm$ 0.2 (1033)	n.d.	<b>91<math>\pm</math>1</b> (384)	<b>89<math>\pm</math>1</b> (121)
% [NAD(P)H] <sub>m</sub>	100 $\pm$ 2 (143)	n.d.	112 $\pm$ 7 (40)	<b>170<math>\pm</math>6</b> (62)
\$ATP: [ATP] <sub>m</sub>	100 $\pm$ 5 (22)	83 $\pm$ 5 (6)	115 $\pm$ 7 (17)	84 $\pm$ 6 (3)
\$ATP: [ATP] <sub>c</sub>	100 $\pm$ 9 (20)	118 $\pm$ 8 (6)	123 $\pm$ 8 (11)	115 $\pm$ 3 (3)
%Calcium: [Ca <sup>2+</sup> ] <sub>c</sub>	100 $\pm$ 4 (32)	109 $\pm$ 5 (23)	109 $\pm$ 5 (21)	106 $\pm$ 6 (20)
%Calcium: ERCa	100 $\pm$ 1 (81)	n.d.	<b>73<math>\pm</math>2</b> (16)	<b>85<math>\pm</math>6</b> (17)
%ROS: Et	100 $\pm$ 1 (526)	<b>196<math>\pm</math>6</b> (93)	<b>151<math>\pm</math>5</b> (94)	<b>222<math>\pm</math>7</b> (147)
%ROS: CM-DCF	100 $\pm$ 2 (412)	n.d.	<b>212<math>\pm</math>15</b> (76)	<b>275<math>\pm</math>20</b> (117)
%ROS: Mitochondrial roGFP1	38 $\pm$ 2 (42)	n.d.	37 $\pm$ 3 (20)	37 $\pm$ 3 (34)
%ROS: Cytosolic roGFP1	15 $\pm$ 1 (23)	n.d.	16 $\pm$ 1 (22)	24 $\pm$ 2 (29)
%ROS: Lipid peroxidation	100 $\pm$ 3 (99)	n.d.	87 $\pm$ 3 (99)	111 $\pm$ 3 (90)
%Shape: Formfactor (F)	100 $\pm$ 1 (1200)	96 $\pm$ 7	<b>117<math>\pm</math>3</b> (214)	<b>120<math>\pm</math>4</b> (69)
%Shape: Mitos per cell (Nc)	100 $\pm$ 2 (1200)	<b>177<math>\pm</math>25</b> (20)	<b>98<math>\pm</math>10</b> (214)	<b>167<math>\pm</math>9</b> (69)
<i>STIMULATED CELL</i>				
%Calcium: peak [Ca <sup>2+</sup> ] <sub>c</sub>	100 $\pm$ 3 (32)	<b>82<math>\pm</math>4</b> (23)	<b>80<math>\pm</math>4</b> (21)	<b>91<math>\pm</math>4</b> (20)
\$Calcium: peak [Ca <sup>2+</sup> ] <sub>m</sub>	100 $\pm$ 2 (11)	n.d.	<b>76<math>\pm</math>5</b> (3)	<b>89<math>\pm</math>3</b> (5)
\$ATP: peak [ATP] <sub>m</sub>	100 $\pm$ 2 (31)	<b>33<math>\pm</math>5</b> (3)	<b>57<math>\pm</math>5</b> (3)	<b>74<math>\pm</math>1</b> (6)
%Calcium: Ca <sup>2+</sup> removal	100 $\pm$ 5 (32)	<b>169<math>\pm</math>12</b> (23)	<b>164<math>\pm</math>10</b> (21)	<b>134<math>\pm</math>6</b> (20)

The data for healthy control (CT) and CI-deficient patient (P) cell lines, except [ATP]<sub>m</sub> and [ATP]<sub>c</sub> in resting cells, was taken from previous studies (summarized in: **Koopman et al., 2007** and **Willemis et al., 2008**). The numbers indicate the designation of the cell line within the Nijmegen Centre for Mitochondrial Disorders.

**CI activity** is expressed as percentage of the lowest value in a collection of control cell lines (**Smeitink et al., 2001**);

**PN**: passage number, an asterisk indicates the passage number after arrival at the Nijmegen Centre for Mitochondrial Disorders.

**Clinical phenotype**: HCEM, hypertrophic cardiomyopathy and encephalomyopathy; L, Leigh Syndrome; LL, Leigh-like syndrome.

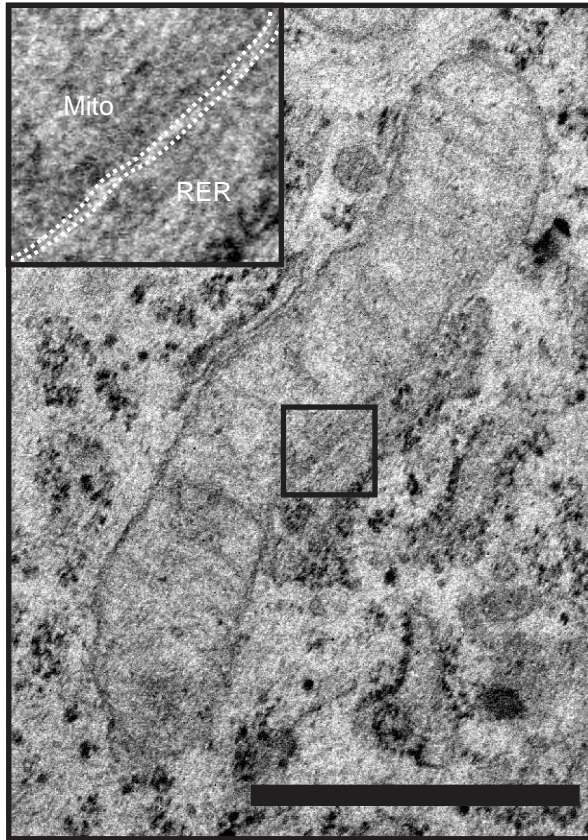
**RESTING CELL**:  $\Delta\Psi$ , mitochondrial membrane potential measured with tetramethyl rhodamine methyl ester (TMRM; **Distelmaier et al., 2008**; **Koopman et al., 2008c**); [NAD(P)H]<sub>m</sub>, mitochondrial NAD(P)H autofluorescence (**Verkaart et al., 2007b**); ATP, see **figure 2** and main text; Calcium: pre-stimulatory fura-2 ratio and ER calcium content (ERCa; **Visch et al., 2006**); ROS: Et, rate of ethidium formation (**Verkaart et al., 2007b**), CM-DCF, rate of chloromethyl dichlorofluorescein formation (**Koopman et al., 2006**; **Verkaart et al., 2007b**); roGFP1, oxidation status (% oxidized) of cytosolic and mitochondria-targeted redox-sensitive GFP (**Verkaart et al., 2007b**); Lipid peroxidation, detected with C11-BODIPY<sup>581/591</sup> (**Verkaart et al., 2007b**), Shape: formfactor is a combined measure of mitochondrial length and degree of branching (**Koopman et al., 2008c**).

**STIMULATED CELL**: Peak values of [Ca<sup>2+</sup>]<sub>c</sub> and [Ca<sup>2+</sup>]<sub>m</sub> measured with fura-2 and mitochondria-targeted aequorin, respectively (**Visch et al., 2006**); Peak ATP measured using mitochondria-targeted luciferase (**Visch et al., 2006**). The rate of cytosolic Ca<sup>2+</sup> removal is determined using the fura-2 signal and given by the time-constant of a mono-exponential fit (larger means slower).

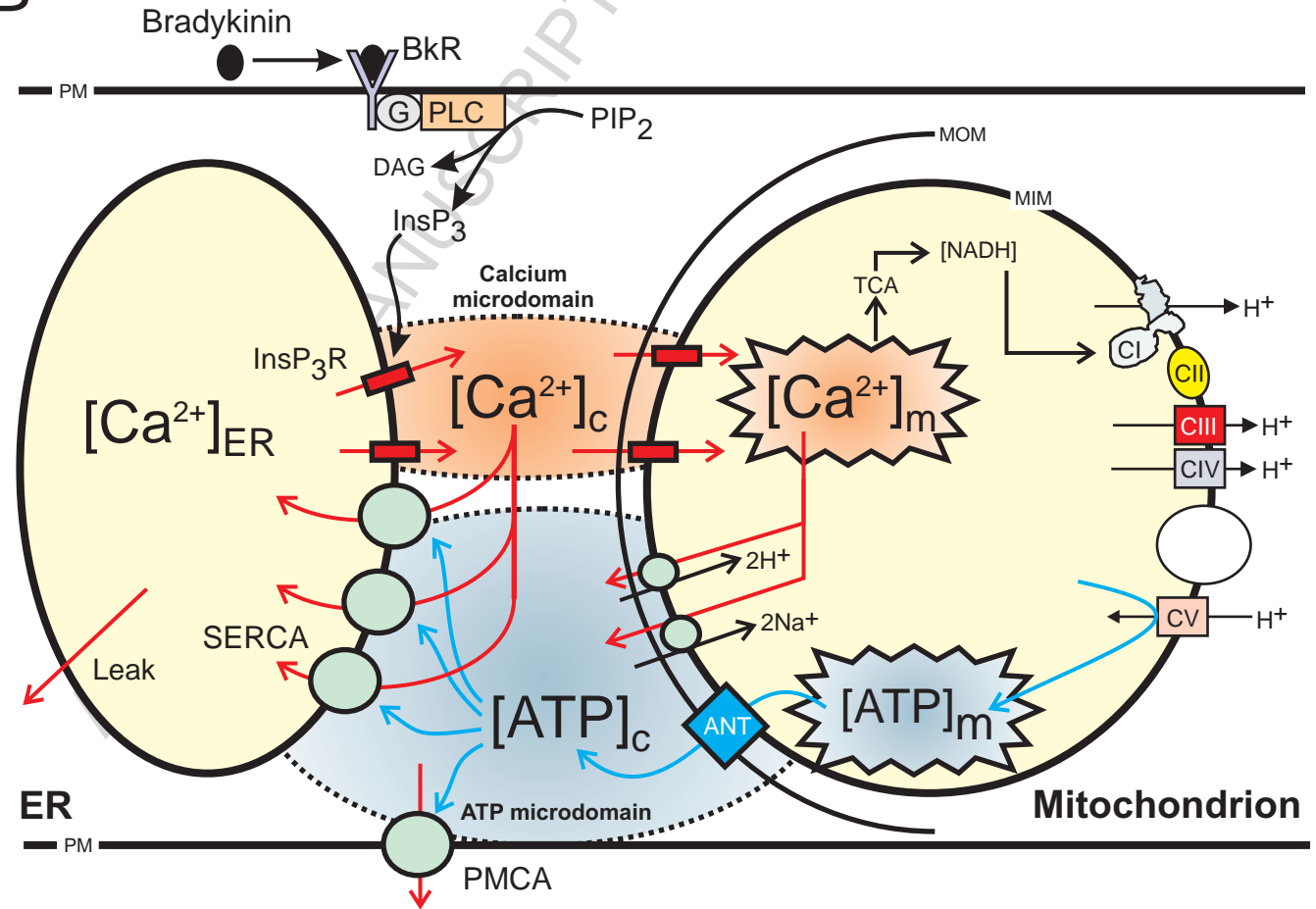
**Statistics**: The data are expressed in percentage of the average control value  $\pm$  standard error. Values in bold are significantly different from control value. %The number between brackets indicates the numbers of cells analyzed on at least 2 different days. \$The number between brackets indicates the number of independent assays on at least 2 different days

**Abbreviations**: CI, complex I; n.a., not appropriate; n.d., not determined; NDUFS, NADH dehydrogenase ubiquinone flavoprotein; Nc, number of mitochondria per cell.

A

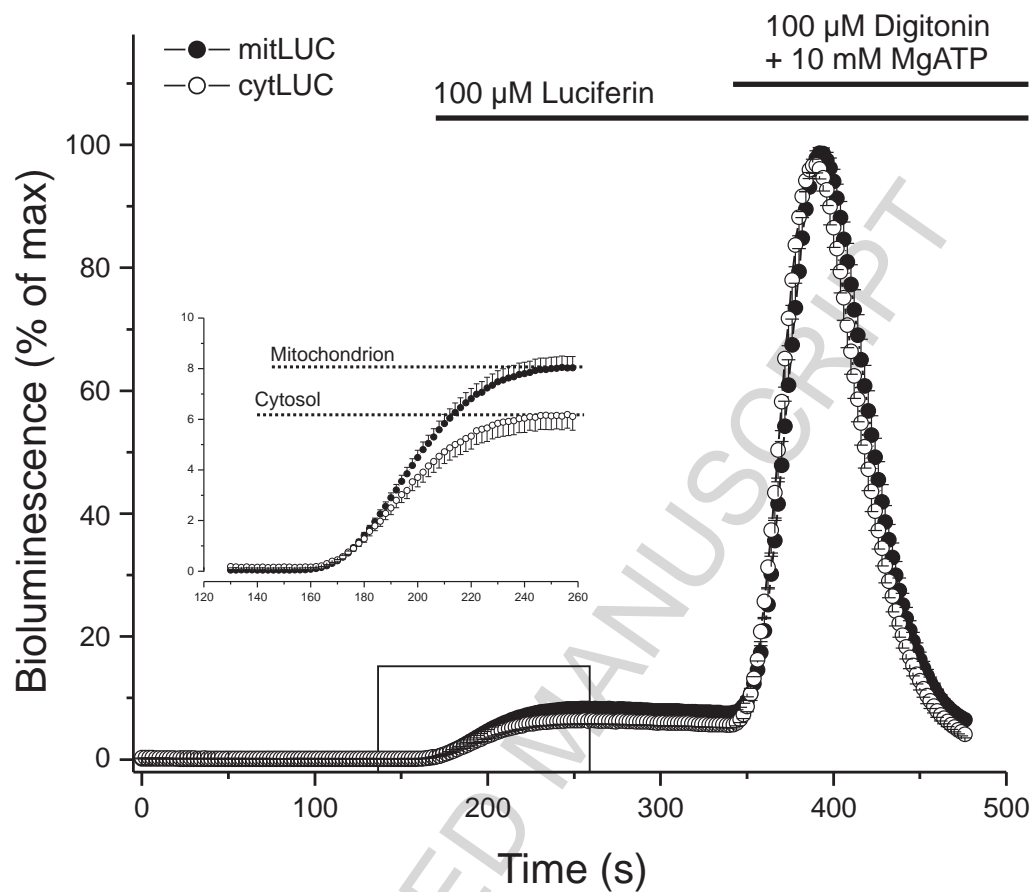


B



Valsecchi et al., Fig.1

A



B

