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OPA1-dependent cristae remodeling disassembles respiratory chain supercomplexes, triggering apoptotic mitochondrial dysfunction

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Sommario

1. Riassunto dell'attività svolta

I mitocondri sono organelli fondamentali per il metabolismo cellulare, per la produzione di energia e per l'omeostasi del calcio (Rizzuto et al., 2000; Danial and Korsmeyer, 2004). Essi, inoltre, hanno un ruolo importante nella morte cellulare (definita anche *apoptosi*) in quanto sono fondamentali nell'amplificazione del segnale di morte indotto da diversi stimoli (Green and Reed, 1998). La loro struttura è molto complessa. Essi sono infatti circondati da una membrana esterna ed una interna. Grazie alle tecniche di microscopia elettronica è stato possibile osservare che la membrana interna presenta delle particolari strutture, denominate *cristae* mitocondriali. Le *cristae* sono dei veri e propri compartimenti distinti della membrana interna e sono separati dallo spazio intermembrana da giunzioni tubulari strette definite *cristae junctions* (Perkins et al., 2001; Frey and Mannella, 2000). I mitocondri sono organelli molto versatili che, grazie ad eventi di fusione e fissione, sono in grado di modificare la propria struttura e morfologia a seconda delle condizioni cellulari. Le proteine che regolano questi eventi appartengono alla famiglia delle dinamine definite *mitochondrial shaping proteins*. OPA1, il cui gene mutato è causa dell'atrofia dominante ottica (ADOA), è l'unica proteina della famiglia delle dinamine localizzata nella membrana interna attualmente conosciuta (Olichon et al., 2002). Nel laboratorio dove ho svolto il mio lavoro di tesi, precedentemente è stato scoperto che OPA1 promuove la fusione mitocondriale cooperando con Mitofusina 1 (MFN1) (Cipolat et al., 2004) e che inoltre possiede un ruolo anti-apoptotico, geneticamente indipendente dalla fusione mitocondriale (Frezza et al., 2006). In particolare è stato dimostrato che OPA1 controlla la morfologia delle *cristae* mantenendone chiuse le giunzioni grazie alla formazione di oligomeri ad alto peso molecolare. Questi oligomeri contengono la forma solubile di OPA1, generata dal taglio proteolitico da parte della proteasi di tipo romboide PARL, e dalle forme di OPA1 ancorate alla membrana mitocondriale. Nelle prime fasi del segnale di apoptosi, la proteina proapoptotica BID, appartenente alla famiglia delle proteine BCL-2, causa la destabilizzazione degli oligomeri di OPA1, provocando un drammatico rimodellamento delle *cristae* (*cristae remodelling*) necessario per la mobilizzazione del citocromo *c* contenuto nelle *cristae*. Al contrario la overespressione di OPA1 stabilizza questi oligomeri e previene la mobilizzazione del citocromo *c* (Frezza et al., 2006).

Inoltre, con tecniche di microscopia elettronica è stato osservato che le cellule deplete di OPA1 presentano una disorganizzazione della struttura delle *cristae*, la cui forma appare irregolare (Frezza et al., 2006). Questa osservazione è stata avvalorata da altre evidenze sperimentali in organismi cellulari differenti che confermano l'importanza di OPA1 nella morfologia delle *cristae* (Olichon et al., 2003; Griparic et al., 2004). Le *cristae* sono strutture importanti per la fisiologia mitocondriale: sono infatti la sede della fosforilazione ossidativa dove i complessi della catena respiratoria sono localizzati. Recentemente, alcune evidenze strutturali e funzionali hanno chiarito

che i singoli complessi della catena respiratoria sono organizzati in macrostrutture dinamiche chiamati supercomplessi della catena respiratoria (RCS) che aumentano l'efficienza del trasporto di elettroni (Acin-Perez et al., 2008). Da questi risultati è stato creato un nuovo modello chiamato "modello plastico" che va ad integrare i modelli "fluido" e "solido" precedentemente disegnati per spiegare l'organizzazione dei complessi della catena respiratoria. Mentre il ruolo del *cristae remodeling* nell'amplificazione del segnale di morte cellulare è stato ben delineato (Scorrano et al., 2002; Frezza et al., 2006; Germain et al., 2005), le sue conseguenze sulle funzioni mitocondriali sono ancora ignote.

Scopo della mia tesi di dottorato è stato quello di capire se il *cristae remodeling* avesse alcun effetto sull'attività e la struttura della catena respiratoria e in particolare sui RCS che sono localizzati proprio sulle *cristae*. I mitocondri però, durante l'apoptosi, vanno incontro non solo al *cristae remodeling* ma ad altri numerosi cambiamenti, inclusi il rilascio di citocromo *c* dalla membrana esterna e l'inattivazione della respirazione mitocondriale da parte di segnali retroattivi. Per poter valutare quali fossero gli effetti specifici del *cristae remodeling* sulla fisiologia mitocondriale, abbiamo dovuto identificare un agente apoptotico che causasse tutti i cambiamenti apoptotici escluso il *cristae remodeling.* Analizzando la sequenza amminoacidica di BID, abbiamo identificato un dominio, corrispondente all'elica α6, che possiede un'elevata omologia con il mastorapan, un peptide contenuto nel veleno delle vespe che è in grado di perturbare fortemente le membrane mitocondriali (Pfeiffer et al., 1995). Per questa sua caratteristica, il mastoparan sembrerebbe un composto naturale in grado di esercitare la stessa perturbazione della struttura delle membrane che avviene durante il *cristae remodeling*. Il mutante di BID nelle due Lisine (K 157, 158) conservate in questo dominio (BID^{KKAA}) è in grado di localizzarsi sui mitocondri così come la sua controparte wt ma è meno efficiente nel causare il rilascio di citocromo *c*. Con esperimenti di crosslinking abbiamo dimostrato che le suddette mutazioni nell' elica α6 non impediscono l'oligomerizzazione di BAK ma riducono la capacità di BID di destabilizzare i complessi ad alto peso molecolare di OPA1. Il risultato è una diminuzione della percentuale di morte cellulare. Per questi motivi, abbiamo utilizzato questo mutante per studiare gli effetti del *cristae remodeling* sulla fisiologia mitocondriale. Abbiamo quindi osservato che BID wild-type (wt) diminuisce in maniera selettiva la respirazione mitocondriale dipendente dal complesso I la quale è strettamente influenzata dall'assemblaggio dei RCS. Dai risultati di Blue Native page (BN PAGE) abbiamo confermato che BID agisce sui RCS bloccando l'assemblaggio dei singoli complessi della catena respiratoria nei RCS, senza interferire direttamente sulla struttura degli stessi complessi. Questi cambiamenti sono abrogati dal mutante di BID che non è in grado di provocare *cristae remodeling* ma sono mantenuti dai mutanti di BID che non attivano l'oligomerizzazione di BAX e BAK. Le basi molecolari che regolano la formazione e la stabilizzazione dei RCS sono a tutt'oggi sconosciuti. L'effetto selettivo del *cristae remodeling* sui RCS ha richiamato la nostra attenzione su OPA1, i cui oligomeri, bersaglio di BID durante l'apoptosi, sono importanti per il controllo della

morfologia delle *cristae* . Per analizzare il ruolo di OPA1 nella formazione strutturale dei RCS, abbiamo utilizzato un approccio genetico e abbiamo analizzato la struttura e l'assemblaggio dei RCS nelle cellule *Opa1*$^{-/-}$. Esperimenti di BN PAGE seguiti da BN seconda dimensione denaturante SDS-PAGE hanno rivelato che la struttura dei RCS, nelle cellule *Opa1*$^{-/-}$, è fortemente compromessa. In particolare, grazie al saggio di assemblaggio dei supercomplessi (Acin-Perez et al., 2008) abbiamo dimostrato che il regolare schema di formazione dei RCS è perturbato nelle cellule *Opa1*$^{-/-}$ e ciò risulta in un' anomala composizione di RCS. Da BN PAGE eseguiti con proteine codificate dal DNA mitocondriale (mtDNA) marcate con isotopi radioattivi, è emerso però che la quantità totale di proteine mitocondriali è minore nelle cellule *Opa1*$^{-/-}$ rispetto alle cellule wt poichè le cellule *Opa1*$^{-/-}$ hanno meno copie di mtDNA. Questa è una caratteristica in comune con le cellule Mfn 1$^{-/-}$,2$^{-/-}$ che invece hanno una corretta biogenesi mitocondriale. Analizzando l'assemblaggio dei RCS nelle cellule Mfn 1$^{-/-}$,2$^{-/-}$ abbiamo confermato che la loro struttura non è alterata. L'importanza fisiologica del mancato assemblaggio dei RCS è stata verificata testando la crescita delle cellule *Opa1*$^{-/-}$
quando obbligate ad utilizzare esclusivamente la respirazione mitocondriale. In queste condizioni la velocità di crescita delle cellule *Opa1*$^{-/-}$ è fortemente rallentata. Lo stesso fenomeno si verifica quando cellule mancanti BAX e BAK sono trasdotte con BID wt ma non quando trasdotte con il mutante KKAA il quale non causa *cristae remodeling.*
In conclusione, i nostri dati confermano che la morfologia mitocondriale è essenziale per la corretta formazione dei RCS e sottolineano l'importanza di OPA1 come regolatore molecolare della struttura dei RCS. Inoltre i nostri risultati approfondiscono la conoscenza attuale del ruolo e degli effetti del *cristae remodeling* durante l'apoptosi, dimostrando che il *cristae remodeling* ha un importante effetto sull'efficienza respiratoria ed è fondamentale nell'induzione delle disfunzioni mitocondriali che si verificano durante l'apoptosi.

2. Summary

Mitochondria are key organelles in intermediatig cellular metabolism, energy production and calcium homeostasis (Rizzuto et al., 2000; Danial and Korsmeyer, 2004). They also integrate and amplify apoptosis induced by several intrinsic stimuli (Green and Reed, 1998). Their structure is extremely complex, being bound by two membranes. The inner membrane (IMM) can be further divided in two distinct compartments, the so called “boundary membrane” and the cristae, separated from the former by narrow tubular junctions. Such a functional versatility and complexity is controlled by a growing family of “mitochondria-shaping” proteins that regulate fusion and fission events of mitochondrial membranes, ultimately affecting the morphology and ultrastructure of the organelle. Optic Atrophy 1 (OPA1), the homologue of *S.cerevisiae* Mgm1p, is the only dynamin-related protein identified in the inner membrane (IM) so far (Olichon et al., 2002). Our laboratory discovered that OPA1 promotes mitochondrial fusion by cooperating with MFN1 (Cipolat et al., 2004), and demonstrated that OPA1 has antiapoptotic activity, genetically distinguishable from its function in mitochondrial fusion (Frezza et al., 2006). In particular we proved that OPA1 keeps the cristae junctions tight by forming oligomers that contain two forms of OPA1, one soluble in the IMS, generated by intramembrane proteolytic cleavage by the rhomboid protease PARL, and the second inserted in the IMM. In the early steps of apoptosis, the pro-apoptotic BH3-only BCL-2 family member BID disrupts these OPA1-containing oligomers and causes a remodeling of cristae structure. Conversely, high levels of OPA1 stabilize them and prevent mobilization of cytochrome c from mitochondria (Frezza et al., 2006). Notably, EM analysis and EM tomography showed that OPA1-depleted cells harbour disorganized cristae whose shape is irregular (Frezza et al., 2006), an observation supported by other experimental evidence in different cellular models that point out the importance of OPA1 in cristae morphology (Olichon et al., 2003; Griparic et al., 2004).

The cristae are key mitochondrial structures: they are the site of oxidative phosphorylation where the complexes of respiratory chain are localized. Recent structural and functional evidence demonstrated that in order to improve the efficiency of electron channelling, the individual respiratory chain complexes are organized into functional and dynamic supramolecular structures referred as supercomplexes (RCS). (Acin-Perez et al., 2008) These evidence gave rise to a novel model called “plasticity model” that integrates the old “fluid” and “solid” models that have been put forward to explain the organization of the electron transport chain . While the role of cristae remodelling in the amplification of the apoptotic cascade has been established (Scorrano et al., 2002; Frezza et al., 2006; Germain et al., 2005) its consequences on mitochondrial function are unknown.

The aim of this PhD thesis was to investigate if apoptotic cristae remodelling had any effect on the activity and the structure of the mitochondrial respiratory chain, and on supercomplexes (RCS),that

are preferentially located in the cristae. Since the multiple changes affect mitochondria during apoptosis, including the release of cytochrome *c* and the activation of feedback circuits that lead to the inactivation of mitochondrial respiration, we needed to identify an inducer of apoptosis that causes all the apoptotic changes except for cristae remodeling. Analyzing the aminoacidic structure of BID, we identify a stretch of aminoacid, corresponding to the α6-helix, that displays high homology with mastoparan, a wasp venom peptide that severely perturbs mitochondrial membranes (Pfeiffer et al., 1995) and that seemed a natural candidate to exert the membrane perturbation of the cristae remodeling. A BID mutant in two conserved Lysines (K157,158) of this domain (BID^{KKAA}) was able to target mitochondria as well as its wt counterpart but was less efficient in inducing cytochrome *c* release. Crosslinking experiments revealed that the mutation in the α6-helix does not impair the oligomerization of BAK, but reduces the ability of BID to distrupt the high molecular weight complexes of OPA1, resulting in a reduction of the apoptotic rate. Thus, we capitalized on this mutant to study the effects of cristae remodeling on mitochondria physiology. Interestingly, wt BID selectively impairs complex I dependent mitochondrial respiration which is higly influenced by RCS assembly. BlueNative page (BN PAGE) confirmed that BID impacts on RCS by preventing the individual complexes from being assembled into supercomplexes, without affecting complexes. These changes were abrogated by the BID mutant that is unable to induce cristae remodeling, but not by other mutants of BID that do not trigger activation of BAX, BAK.

Since the molecular basis of the assembly and stabilization of RCS are unknown, the selective impairment of RCS by cristae remodeling pointed our attention to the role of OPA1, whose oligomers are targets of BID and control cristae bioegnesis. We turned to a genetic approach and analyzed the structure and the assembly of RCS in *Opa1*$^{-/-}$ cells. First dimension BN PAGE and SDS-second dimension page revealed that the structure of RCS in *Opa1* deficient cells is dramatically affected. In particular a RCS assembly assay (Acin-Perez et al., 2008) confirmed that the ordered pattern of assembly is perturbed in *Opa1*$^{-/-}$ cells, resulting in anomalous composition of RCS. BN PAGE performed with radiolabeled mt-DNA proteins revealed that the overall amount of mitochondrial protein is less in *Opa1*$^{-/-}$ than in wt cells and it correlates with the fact that *Opa1*$^{-/-}$ cells have less mt-DNA copy number a feature shared also by *Mfn1*$^{-/-}$*,2*$^{-/-}$ cells that on the contrary display organized cristae. Of note, BN PAGE and assembly assay demonstrated that the structure of RCS is not affected in *Mfn1*$^{-/-}$*,2*$^{-/-}$ cells. The physiological importance of RCS disassembly is substantiated by an impairment of the growth rate of *Opa1*$^{-/-}$ cells or of cells lacking *Bak* and *Bax* expressing wt, but not KKAA BID when energy production cannot bypass mitochondrial respiration. In conclusion, our data indicate that the shape of the cristae is essential for the assembly of the RCS and underline OPA1 as molecular regulator of the RCS structure. Moreover our results deepen the role of cristae remodelling during apoptosis, demonstrating that cristae remodelling affects mitochondrial respiratory efficiency and hence the program of mitochondrial dysfunction during apoptosis.

3. Introduction

Mitochondria are fundamental organelles in life and death of eukaryotic cells. They are the main site of energy production and they have a central position in the programmed cell death pathway. Moreover, they are involved in many others processes, such as Ca^{2+} homeostasis, cellular differentiation, control of cell cycle and growth, amplification of signaling cascades. Finally, mitochondria are involved in several human diseases, including neurodegenerative disorders and cancer, and may play a role in aging processes.

The structure of these organelles is very elaborate and supports them in these multiple functions. According to the needs of cells, mitochondria could be organized in networks of interconnected, fused mitochondria or undergo fission and be retrieved as single, small organelles in the cytoplasm. Similarly, the ultrastructure of mitochondria is extremely complex, with the organelle bound by two distinct membrane: the outer membrane (OMM) and the inner membrane (IMM). The IMM is organized in distinct compartments, the peripheral inner membrane and the *cristae* that are separated from the peripheral inner membrane by narrow tubular junction. The cristae are key mitochondrial structures: they are the site of oxidative phosphorylation where the complexes of respiratory chain are localized. Recent structural and functional evidence demonstrated that the respiratory chain complexes are organized into functional and dynamic supramolecular structures referred as supercomplexes (RCS) in order to improve the efficiency of electron channelling. During apoptosis, the so called cristae remodelling process induces a dramatic reorganization of the IMM in order to induce the mobilization of cytochrome *c.* In this thesis we analyzed the effect of cristae remodelling and the importance of cristae shape on the structure of RCS, focusing on the role of the mitochondria-shaping protein OPA1. This introduction will therefore address specifically our knowledge of the interplay between mitochondrial structure and function in cell life and death.

3.1. Mitochondria

Mitochondria are organelles that play a crucial role in many cell pathways. They were described for the first time in 1857 by the anatomist Rudolf Albrecht von Koelliker who called them “sarcosome”. Mitochondria are often defined as “power house” of the cells. They produce most of ATP needed for endoergonic processes and convey it to the sites of greater energy demand. Moreover they shape and participate in complex signalling processes such as cytosolic Ca^{2+} transient (Jouaville et al., 1995). They take part in apoptosis integrating diverse stimuli by releasing cofactors that trigger the activation in the cytosol of effectors caspases, leading to cell death (Wang, 2001). Defects in any of these processes can be detrimental for the cell: several pathological conditions including

cancer or neurodegenerative disease are indeed consequences of, or are aggravated by mitochondrial dysfunction (Schapira, 2000).

Mitochondria are the only organelle that possesses its own DNA; in vertebrates mitochondrial DNA (mtDNA) consists of a double stranded covalently close circular DNA molecule of about 16.5 kb. The mtDNA encodes 13 mRNAs for subunits of the oxidative phosphorylation complexes (OXPHOS) (Fernandez-Silva et al., 2003). Proteins of mitochondrial origin are translated on mitochondria ribosome bound to the matrix site of the inner membrane and then they are addressed to the proper compartment. Take together, these features support the endosymbiontic theory, developed in 1971 by Margulis. (Margulis, 1971). According to this theory, mitochondria have an ancestral extracellular origin, deriving from primordial rickettsia-like intracellular bacteria that colonized the eukaryotic cell and become essential for its life. Mutations in mtDNA are associated with a number of genetic, multisystemic diseases that highlight the importance of this organelle in physiology of multiple organs.

3.2. Mitochondrial shape and dynamics

Mitochondrial shape in living cells is very heterogenous and can range small spheres to interconnected tubules (Bereiter-Hahn and Voth, 1994). The different shapes of mitochondria were already observed in early times by cytologists who looked at this organelle under the light microscope. Noticing that mitochondrial morphology was heterogeneous, they accordingly christened this organelle ‘mitochondrion’, a combination of the Greek words for ‘thread” and “grain’. The morphological plasticity of mitochondria results from the ability of this organelle to undergo fusion and fission. Real-time imaging reveals that individual mitochondrial tubules continuously move back and forth along their long axes on radial tracks. Occasionally, two mitochondrial tubules encounter each other and fuse, end to end or head to site (Chen et al., 2003). On the other hand, these tubules can also undergo fission events, giving rise to two or more mitochondrial units. It is important to note that mitochondrial fusion and fission are complicated processes, being mitochondria bound by two membranes. Thus, any mechanism of fusion and fission should take into account that the coordinate fusion or division of four lipid bilayers is required to complete the process.

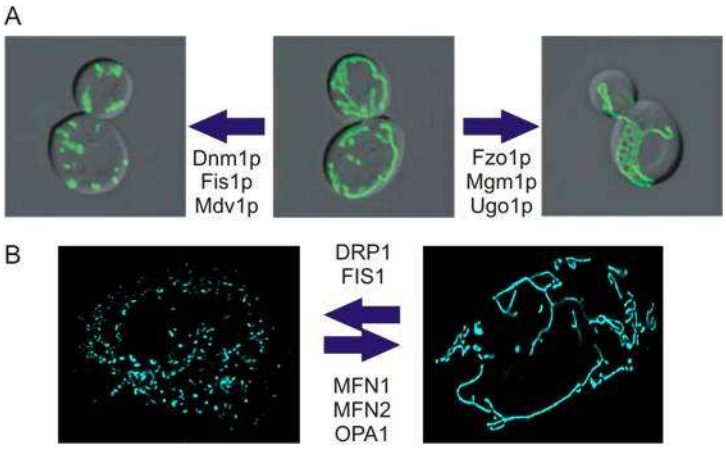

Figure 1: **Fusion and Fission of mitochondrial network.** (A) cartoon depicting the structure of mitochondrial network in *S.cerevisiae* and the relative mitochondrial shaping proteins regulating fusion and fission processes. (B) cartoon depicting the different shape of mitochondrial network in mammals and the dynamin related proteins that regulate these processes.

3.2.1. Mitochondria-shaping proteins: Proteins involved in mitochondrial fusion

3.2.1.1. Fzo/Mitofusin-1-2

Proteins involved in mitochondrial morphology have been uncovered in the recent years. The first mediator of mitochondrial fusion to be identified was the *D. melanogaster* Fuzzy onions 1 protein (Fzo1p), a large transmembrane (TM) guanosine triphosphatase (GTPase) required for the formation of the giant mitochondrial derivative during spermatogenesis (Hales and Fuller, 1997). The *S. cerevisae* ortholog of Fzo1p mediates mitochondrial fusion events during mitotic growth and mating and is required for long-term maintenance of mitochondrial deoxyribonucleic acid (mtDNA) (Hermann et al., 1998) In mammals, two Fzo1p homologues, Mitofusin (MFN)-1 and -2, are widely expressed in many tissues (Rojo et al., 2002; Santel et al., 2003; Eura et al., 2003). MFN1 and -2 display high (81%) identity, similar topologies, both residing in the OM (Santel and Fuller, 2001; Legros et al., 2002; Rojo et al., 2002; Chen et al., 2003; Santel et al., 2003). MFN1 and -2 possess a GTPase domain and a coiled coil domain located at the N-terminus of the proteins, protruding towards the cytosol. Two TM regions form a U-shaped membrane anchor, ending in a cytosolic, C-terminal coiled coil motif (Rojo et al., 2002; Koshiba et al., 2004; Santel, 2006). The coiled coil is a widespread helical structural motif functioning as oligomerization domain (Oakley and Hollenbeck, 2001). In the case of MFNs, two molecules on opposing membranes can bind in *trans* to bridge mitochondria, maintaining a distance of 95 Å between the two membranes (Koshiba et al., 2004). *In silico* analysis of MFN2 reveals that this protein also has a proline-rich-domain between aminoacids 576 and 590 which is poorly conserved in MFN1 and Fzo1p. Proline-rich domains are involved in the binding to other proteins (Kay et al., 2000).It has been recently reported that MFN2 forms high molecular weight complexes with stomatin-like protein 2 (Stoml2), a novel protein

associated to the IM, facing the IMS. The function of this protein remains still unknown, however the protein does not seem to have a role in regulating mitochondrial morphology (Hajek et al., 2007). Despite their high homology, MFNs are not functionally redundant. First, MFN1 has a higher GTPase activity than MFN2, although its affinity for GTP is lower (Ishihara et al., 2004). Accordingly, direct measurments of mitochondrial fusion rates in *Mfn1*$^{-/-}$ and *Mfn2*$^{-/-}$ cells showed that cells containing only MFN1 retain more fusion activity than those that contain only MFN2 (Chen et al., 2003). Finally, MFN1 but not MFN2 is essential to allow OPA1-dependent mitochondrial fusion in embryonic fibroblasts (Cipolat et al., 2004).

Along this line, our laboratory suggested a role for MFN2 beyond fusion, in regulating the shape of the endoplasmic reticulum (ER), and as the first molecularly identified tether between mitochondria and the ER. MFN2 localizes not only on mitochondria, but it is highly enriched at the level of the mitochondria-ER interface and present (albeit to a lesser extent) at the ER. Genetic ablation of MFN2 disrupts the structure of ER and loosens the ER-mitochondria interaction, thereby reducing mitochondrial Ca^{2+} uptake dependent on the generation of Ca2+ microdomains between ER and mitochondria (de Brito and Scorrano, 2008). Moreover, MFN2 binds and inhibits the proto-oncogene Ras. The modulation of Ras signalling does not influence the mitochondria-ER interaction, underlining the principal role of MFN2 in shaping and juxtaposition of these two organelles (de Brito and Scorrano, 2009). In conclusion MFN1 and 2 seem to play different role in mitochondrial physiology, with MFN1 that (in cooperation with OPA1) exquisitely regulates mitochondrial fusion and MFN2 that plays a role in maintaining mitochondria-ER interactions, ultimately impacting on mitochondrial metabolism, apoptosis and even progression trough cell cycle.

3.2.1.2. Mgm1p/ Msp1p/ OPA1

Optic atrophy 1 (OPA1) is a dynamin-related protein located in the IMM. Mgm1p, the yeast homologue of OPA1, has been identified in a genetic screen for nuclear genes required for the maintenance of mtDNA in the budding yeast *S. cerevisae* (Jones and Fangman, 1992). Years later, Pelloquin and colleagues isolated Msp1p, the *S. pombe* orthologue (Pelloquin et al., 1999). The human gene *OPA1* was identified in 2000 by two independent groups (Delettre et al., 2000; Alexander et al., 2000). A more detailed analysis showed that Mgm1p, Msp1p and OPA1 are localized in the IMS, tightly associated with the IMM (Sesaki et al., 2003; Wong et al., 2003; Cortopassi and Wong, 1999). These proteins, albeit they display a sequence identity of approximately 20%, maintain a highly conserved secondary structure, consisting of two predicted coiled coils, one N-terminal to the GTPase domain and the other at the C-terminus. The C-terminal coiled coil domain of OPA1 may function as a GTPase effector domain (GED). On its N-terminal, OPA1 possesses a mitochondrial targeting sequence that targets the protein to mitochondria (Satoh et al., 2003). Studies in yeast show that MTS of Mgm1p is cleaved by the mitochondrial processing peptidase (MPP) upon import (Satoh et al., 2003). The functional analysis of Mgm1p

and Msp1p reveals that both proteins are required for the maintenance of fusion-competent mitochondria in

S. cerevisae and *pombe*. Mgm1p forms a complex together with Fzo1p which participates in the coordinated fusion of the IMM and OMM (Wong et al., 2003). The high degree of secondary structure conservation suggests that the function of OPA1 is conserved in mammals. On the other hand, it was less clear whether OPA1 played a role in fission, rather than in fusion of mitochondria. Overexpression studies indeed showed that high levels of OPA1 can drive fragmentation of the mitochondrial reticulum (Misaka et al., 2002; Olichon et al., 2003). However, overexpression of OPA1 or its downregulation by siRNA in mouse embryonic fibroblasts showed a linear relationship between OPA1 levels and mitochondrial fusion (Cipolat et al., 2004). We will discuss in detailed and separated chapter the role of OPA1 in disease, the regulation of OPA1 by proteolytic processing, the role of OPA1 in the structure of the *cristae* and in mitochondria-dependent apoptosis

3.2.1.3. Ugo1

A third mitochondrial fusion protein named Ugo1p has only been identified in fungi (Ugo means fusion in Japanese) (Sesaki and Jensen, 2001). Ugo1p is a member of transport protein family: it contains three transmembrane segment in the middle of the protein and exists as a dimer. It has been demonstrated that it is involved in the outer and inner membrane fusion after the tethering of membrane. In particular seems to be involved in the regulation of membrane lipid composition (Hoppins et al., 2009)

3.2.2. Mitochondrial shaping proteins: Proteins involved in mitochondrial fission

3.2.2.1. Dnm1p/DLP1/DRP1

The two proteins FIS1 and DRP1 are required for mitochondrial fission in mammals. DRP1 belongs to the dynamin superfamily. Dynamins are large GTPase that participates in membrane scission in multiple endocytic and secretory organelles (Praefcke and McMahon, 2004). The dynamin-like-protein (Dlp)1p in yeast, DRP1 in *C. elegans*, and DLP1/DRP1 in mammals are homologues. DRP1 exists largely in a cytosolic pool, but a fraction is found in spots on mitochondria at sites of constriction (Labrousse et al., 1999; Smirnova et al., 2001). DRP1 contains a dynamin-like-central domain and a C-terminal GTPase effector domain (GED), in addition to its N-terminal GTPase. Intramolecular interaction between the GTPase and GED regions appear to be required for full GTPase at fission activities (Zhu et al., 2004). DRP1 can oligomerize, *in vitro*, into ring-like structures and intermolecular oligomerization is observed at membrane constriction sites. Given

these similarities with dynamin, DRP1 has been proposed to couple GTP hydrolysis with mitochondrial membrane constriction and fission (Hinshaw, 1999; Smirnova et al., 2001).

3.2.2.2. Fis1p/hFis1

Fis1 is an outer membrane protein evenly distributed on the surface of mitochondria (James et al., 2003). Its N-terminal domain is exposed to the cytoplasm and forms a tetratricopeptide (TPR)-like fold (Suzuki et al., 2003). The C-terminal domain of Fi1 possesses a predicted TM domain and a short stretch of aminoacids facing the IMS. Fis1 is thought to recruit DRP1 to punctuate structures on mitochondria during mitochondrial fission. It is therefore considered the limiting factor in the fission reaction (Stojanovski et al., 2004). During assembly of the yeast mitochondrial fission complex, the outer membrane protein Fis1 recruits the dynamin-related GTPase Dnm1 to mitochondria. Moreover it has been shown that the N-terminal Fis1 arm acts in an autoinhibitory manner to regulate access to a binding pocket that is evolutionarily conserved for binding the dynamin-like GTPase Dnm1 (Wells et al., 2007). It can be speculated that this autoinhibitory function of Fis1 arm is conserved also in mammalian cells and regulate binding of hFIS1 with Drp1.

3.3. Roles and mechanisms of mitochondrial fusion and fission

Mitochondria undergo fusion and fission cycles in order to modulate their morphology, distribution and function according to the needs of the cells. First fusion and fission control the shape, length and number of mitochondria. The balance of these two opposite events controls mitochondria morphology. Second, fusion and fission allow mitochondria to exchange lipid membranes and intramitochondrial content. Such exchange is probably crucial to maintain the health of the mitochondrial population. Indeed, when mitochondrial fusion is abolished a large fraction of mitochondria looses nucleoids (Chen et al., 2005). In addition to mtDNA, other components, such as substrates, metabolites, specific lipids and protein can be restored in defective mitochondria during fusion (Muster et al., 2010). Third, the shape of mitochondria affects the ability of cells to distribute the mitochondria to specific subcellular locations such as dendrites (Li et al., 2004) and the uropod of migrating leukocytes (Campello et al., 2006). Mitochondrial fission facilitates apoptosis by regulating the release of intermembrane- space protein into the cytosol (Cereghetti and Scorrano, 2006). Mitochondria participate in the regulation of Ca^{2+} signalling and this process relies on the relative position of mitochondria in the cytosol, as well as on their juxtaposition to the ER (Rizzuto et al., 2000). It is therefore conceivable that changes in mitochondrial shape influence mitochondrial participation in the Ca^{2+} game. In general the regulation of fusion and fission occurs by modification of the proteins involved in these processes such as sumoylation, phosphorylation and ubiquitination, but there is also regulation at the level of transcripts. Mitochondrial fission in

mammalian cells seems to follow the same mechanism as in yeast. As in yeast, it has been shown that DRP1 is recruited to spots on mitochondria and it seems that constriction of the membranes takes place via interaction with Fis1, since it has been shown that recombinant DRP1 and recombinant Fis1 interact *in vitro* (Yoon et al., 2003). However, this association has never been shown *in vivo* and reduction of FIS1 levels by siRNA does not disrupt DRP1 localization to mitochondria (Lee et al., 2004), even if the residual level of Fis1 could still be sufficient to recruit DRP1 to mitochondria. DRP1-dependent mitochondrial fragmentation is controlled by phosphorylation at Serine 616 by Cdk1 and dephosphorylation at Serine 637 by the Ca^{2+}-dependent phosphatase calcineurin (Chang and Blackstone, 2007; Jahani-Asl and Slack, 2007; Taguchi et al., 2007). DRP1 forms a complex with calcineurin and cyclophilin A in the cytosol. When calcineurin is activated by an increase of cytosolic Ca^{2+} (such as the one that occurs following mitochondrial depolarization), it dephosphorylates DRP1, which translocates to mitochondria and induces fission of the organelle (Cereghetti et al., 2008). Mitochondrial DRP1 is further regulated by sumoylation, that stabilizes the pool of DRP1 retrieved on mitochondria (Zunino et al., 2007) . Sumoylation is a process that involves the covalent binding of the small protein SUMO to the substrate, protecting it from binding to ubiquitin and therefore from degradation by the proteasome (McConnell and Yaffe, 1993). Along this line, the ubiquitin ligase of the OM MARCH-V regulates targeting of DRP1 for degradation (Karbowski et al., 2007). Fusion of mammalian mitochondria is thought to occur in a similar way as in yeast. The mammalian orthologues of Fzo1p, MFN1 and MFN2, are believed to dock two juxtaposed mitochondria *via* their coiled coil domains (Koshiba et al., 2004). However, MFN2 seems to have a different role from MFN1. It has been shown that MFN1 has a higher GTPase activity than MFN2, although its affinity for GTP is lower (Ishihara et al., 2004). In agreement with this, MFN1 exhibits a higher capacity in inducing fusion. But how is OMM fusion coordinated with IMM fusion? In yeast a multimolecular complex of Mgm1p, Ugo1p and Fzo1p apparently coordinates fusion of the two membranes. A functional requirement for mitochondrial fusion is an intact IMM potential but is independent of a functional cytoskeleton (Mattenberger et al., 2003). Taking advantage of the yeast model, the recent recapitulation of mitochondrial fusion *in vitro* has allowed the fusion process to be dissected into two mechanistically distinct, resolvable steps: OMM fusion and IMM fusion. OMM fusion requires homotypic trans interactions of Fzo1p, the proton-gradient component of the inner membrane electrical potential, and low levels of GTP hydrolysis. Fusion of IMM requires the electrical component of the inner membrane electrical potential and elevated levels of GTP hydrolysis.

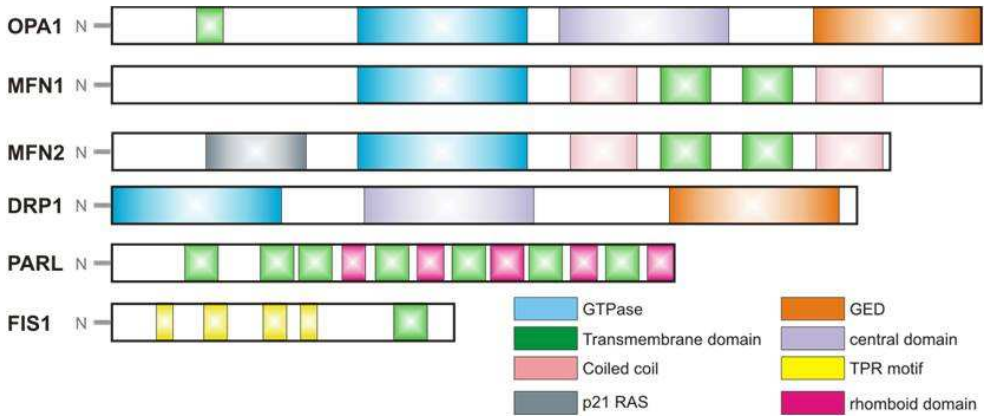

Figure 2: Mitochondria-shaping proteins in mammals and their domains

3.4. Mitochondrial ultrastructure

In the Fifties, the nascent technique of electron microscopy allowed to inspect the structure of cellular organelles. In 1952, Palade described mitochondria as organelles with an outer and inner membrane, a continuous surface which is folded in ridges called “cristae mitochondriales”. This was called “baffle model” and it defined the cristae as invaginations of the inner membrane with large openings to the intermembrane space on one side and protruding across the matrix in the other side (Palade, 1952). Few years later Sjostrand proposed that the cristae are formed by the formation of septa of the inner membrane (“septa model”) (Sjostrand, 1953). The current model of mitochondrial ultrastructure was described by Mannella and colleagues in the 1990s (Mannella et al., 1994), by applying electron tomography on isolated rat liver mitochondria.

The resulting tomograms showed that cristae are not simple invagination of the inner membrane as depicted by Palade but rather they are distinct compartment of it. High-voltage electron tomography coupled with three-dimensional image reconstruction *in situ* in several different tissues, demonstrated that *cristae* are separated from the inner boundary membrane. They are shaped like bags and connected by narrow tubular junctions (with a diameter of approximately 28 nm) to the thin intermembrane space (Perkins et al., 2001; Frey and Mannella, 2000).

In contrast to the standard baffle model for mitochondrial structure, this new structural organization strongly suggest that diffusion between internal compartments is restricted, which has profound functional implications.

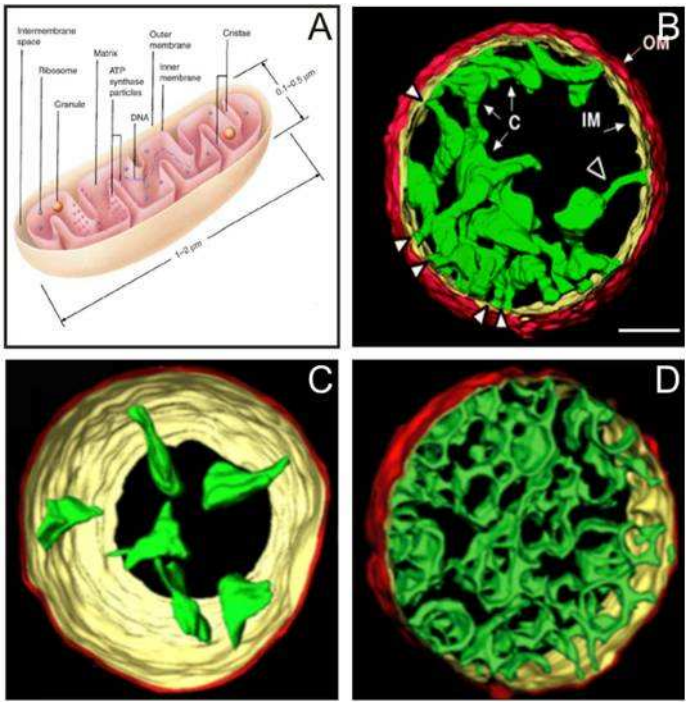

Figure 3: Mitochondrial ultrastructure. (A) A representation of the Baffle model adapted from (Frey and Mannella, 2000). (B) Three –dimensional reconstructions of isolated rat liver mitochondria obtained by high-voltage electron microscopy tomography OM: outer membrane, IM: inner membrane, C: isolated cristae, arrowheads point to narrow tubular regions that connect cristae to pehriphery and to each other. Bar, 0.4µm. Adapted from (Frey and Mannella, 2000). (C-D) Rapresentative surface-rendered views of electron microscopy tomography reconstractions of class I and class II mitochondria. OM is depicted in red, inner boundary membrane in yellow and cristae in green.

Biochemical fractionation of submitochondrial membranes and the localization of marker proteins in isolated mitochondria using electron tomography allow the description of a more complete picture of the structure and compartmentalization of mitochondria.

- Outer membrane

The outer membrane separates mitochondria from the cytosol and it is the platform where all the proteins that allow mitochondria to communicate with the cell are located. The most abundant protein of OM is the voltage dependent anion channel (VDAC) that facilitates the exchange of ions and molecules between the cytosol and the mitochondria (Colombini et al., 1996). In addition to its role in ion and metabolite flux, VDAC2 has been implied in apoptosis, either by inhibiting the proapoptotic protein BAK (Roy et al., 2009a). Other proteins that localize in the outer membrane include the recently discovered MTCH2/MIMP that is a molecular receptor for tBID (Zaltsman et al., 2010). The role of this protein is commented in detail in the "*Research highlight*" attached in section 2.9.4. The OMM is enriched by proteins of the import machinery such as TOM20,TOM22 and TOM70 that act as receptors for the proteins that have to be imported into mitochondria (Pfanner and Wiedemann, 2002). As it has been mentioned in the previous chapter, MFN1, 2 and Fis1 are also located in the outer membrane.

- Inner membrane

The inner boundary membrane is decorated by proteins that mediate the translocation of the protein into the matrix, such as the TIM complexes (Pfanner and Meijer, 1997), and proteins that, such as Oxa1, guide the right localization of proteins into the membrane (Herrmann and Neupert, 2003) .

- Cristae

The cristae are important structure for the mitochondria. They are defined as the site of oxidative phosphorylation (Gilkerson et al., 2003). Accordingly, the cristae membrane contains the proteins involved in the oxidative phosphorylation. Recently it has been demonstrated that the complexes of respiratory chain are assembled into supercomplexes on the membrane surface of the cristae (Acin-Perez et al., 2008). The ATPsynthase dimers are at the apex of the cristae (Schagger and Pfeiffer, 2000a; Wittig et al., 2006). Because of this topical organization, the major part of the cytochrome *c* is located into the cristae.

Such a highly defined compartmentalization suggests that the mitochondria cristae are special substructure that ensure optimal condition for the ATP production limiting the diffusion of metabolities such as protons or ADP during respiration (Demongeot et al., 2007). In turn the cristae shape and density could be modify by the respiratory state of mitochondria (Hackenbrock, 1968; Hackenbrock et al., 1980). Moreover the major part of cytochrome *c* is stored into the cristae (Scorrano et al., 2002). This corroborates the hypothesis that cristae are special compartments involved in respiration and also suggests that cristae might have an important role during apoptosis, when cytochrome *c* must be released into the cytoplasm. The cristae shape is maintained by the cristae junctions that represent a functional barrier between the cristae space and the inter membrane space. Many factors could affect the cristae shape and modulate the cristae junction openings, ultimately impacting on the diffusion of metabolites and of proteins such as cytochrome *c*. We will discuss later the details of these processes in the chapter dealing with apoptosis.

3.5. Proteins and lipids involved in cristae morphology

3.5.1. Prohibitins

Prohibitins were identified for the first time as negative regulators of cell proliferation since their ablation leads to an antiproliferative effect. Two members of the prohibitin family protein, PHB1 and PHB2 are found to be localized preferentially to the mitochondria, anchored to the inner membrane facing the inter membrane space with the carboxy terminal (Berger and Yaffe, 1998; Merkwirth et al., 2008). Protein structure analysis performed in different experimental models revealed that both proteins are present in high-molecular weight complexes in the inner membrane of the mitochondria (Artal-Sanz et al., 2003; Nijtmans et al., 2000). Recently it has been demonstrated

that they form ring-like structure that include multiple PHB1 and PHB2 subunits in the inner membrane of mitochondria (Tatsuta et al., 2005). The functional role of prohibitins has been linked to many biological aspects of mitochondria. Studies have identify PHB1 and 2 as component of mitochondria nucleoids, playing a role in mtDNA mainteinance (Bogenhagen et al., 2003). Moreover, depletion of Phbs induces cellular senescence and reduction of lifespan in yeast and *C. elegans*, demonstrating that Prohibitins are involved in mitochondria dependent ageing (Artal-Sanz and Tavernarakis, 2009; Coates et al., 2001). Depletion of Phbs also impacts on mitochondrial morphology. Prohibitins deficient cells display a fragmented mitochondria network (Merkwirth et al., 2008), as a consequence of the impairment of the inner membrane fusion. Besides, the loss of prohibitins affects the ultrastructure of mitochondria. The cristae morphogenesis is impaired and their shape looks vesicular (Merkwirth et al., 2008). The alteration in cristae shape renders the cells more prone to apoptosis. At the molecular level, aberrant processing of OPA1 could be identified as the underlying cause: in cells lacking *Phbs,* the long form of OPA1 is destabilized and degraded faster (Merkwirth et al., 2008). Prohibitins also interact with unassembled respiratory-chain subunits suggesting that they could have chaperone activity (Nijtmans et al., 2000). Moreover, they interact also with lipids. For these reasons it has been hypothesized that prohibitins could act also as scaffold proteins that define mitochondrial subcompartements of the inner membrane.

3.5.2. Mitofilin

Mitofilin is one of the most abundant mitochondrial proteins, originally identified in the heart (Taylor et al., 2003). Two alternatively spliced variants produce two different proteins product of 88 and 90 KDa. Mitofilin has a predicted membrane anchor and coiled-coil domains with an amino-terminal transmembrane domain with the majority of the protein is extruding into the intermembrane space (Gieffers et al., 1997). Down-regulation of mitofilin in HeLa cells by siRNA results in the formation of concentric onion-like inner mitochondrial membranes (John et al., 2005) The mitofilin-deficient mitochondria tend to form progressively larger membrane swirls, which were analyzed by electron tomography. The larger IMM structures were found to be composed of a complex, interconnected network of membranes totally lacking tubular connections to each other or to the peripheral inner membrane. John et al. proposed that mitofilin's physiological role is to maintain normal cristae morphology, in particular that they regulate the formation or the stabilization of cristae junctions. Recent experimental findings showed that the function of Mitofilin is probably mediated by the interaction with others proteins. In particular it has been demonstrated that Mitofilin interacts with Distrupted-in-schizophrenia 1 (DISC1) (Park et al., 2010) modulating mitochondrial NADH dehydrogenase activity and Ca^{2+} signaling. Moreover the lack of DISC1 affects the stability of Mitofilin. Mitofilin interacts also with the IM proteins, PKA target, ChChd3 and OPA1 (Darshi et al.,

2010). The disruption of this protein complex results in mitochondrial bioenergetics defects and affects the structure of the cristae junction.

3.5.3. Cardiolipin

Cardiolipin (CL) is an anionic phospholipid predominantly found in mitochondrial membranes. It is located in the inner membrane and in particular microdomains called contact site between the outer and the inner membrane (Ardail et al., 1990). CL is able to interact by non-covalent bonds with protein including cytochrome *c* (Tyurin et al., 2007), complexes of the respiratory chain (Zhang et al., 2002) and proteins involved with the apoptotic machinery like BID (Lutter et al., 2000; Gonzalvez et al., 2005; Kim et al., 2004) and caspase 8 (Gonzalvez et al., 2008). Moreover, in yeast, CL plays an important role in the oligomerization of Mgm1. These indications support the idea that CL is a protein scaffold that participates in the modulation of the correct architecture of the cristae. Moreover CL shows an intrinsic curvature and is able to stabilize the geometry of curved regions of membrane by forming clusters (Huang et al., 2006). Experiments performed with giant unilamellar vescicles demonstrated that when they were subjected to a brief-impulse of acid, they can form cristae-like invaginations and this processes is dependent only on CL. The ability of CL to be a proton buffer, is probably implicated in the formation of mitochondrial cristae (Khalifat et al., 2008). The importance of CL in the definition of cristae structure is underlined in Barth syndrome, a genetic disease caused by a mutation in *Tafazzin* gene, an enzyme involved in the maturation of CL (Schlame and Ren, 2006). Mitochondria of patients affected by this disease have 80% less of mature CL and they show severe defects in their ultrastructure as well as in the supercomplexes of the respiratory chain (McKenzie et al., 2006).

3.5.4. ATP synthase

The mitochondrial F_1F_0-ATP synthase complex has been strongly implicated in contributing to the curvature of the inner membrane as a result of its oligomerization. Allen showed that F1 complexes are arranged as a double row of particles along the full length of the helically shaped tubular cristae in *Paramecium multimicronucleatum* (Allen et al., 1989). Based on these observations, he proposed that the double-cone shape of ATP synthase dimers are involved in the formation of tubular cristae upon association of additional complexes during mitochondrial biogenesis.

Interestingly, Paumard et al. have shown that mutations in ATP synthase subunit *e* and *g* inhibit formation of the ATP synthase supermolecular complexes (the first step in formation of larger oligomers) and results in appearance of concentric onion-like cristae (Paumard et al., 2002). Single-particle electron microscopic studies revealed that F_1F_0-ATP synthase

forms oligomers in “ribbons” at the tip of mitochondrial cristae (Minauro-Sanmiguel et al., 2005; Dudkina et al., 2005). However Rak and collegues demonstrated that a mitochondrial DNA mutation in the gene encoding subunit 6 (F_0 domain) affects the activity of the ATP synthase but does not have any effects on mitochondrial cristae structure, indicating that neither Atp6p nor the ATP synthase activity is crucial for cristae generation (Rak et al., 2007). More recently a role in the cristae structure has been suggested for IF1. IF1 is the inhibitor factor of reversal F_1F_0-ATP synthase and it is involved in the regulation of ATPase activity that occurs usually after inhibition of respiration, during ischemia, or hypoxia. Its overexpression increases the oligomerization of ATPsynthase and the cristae density (Campanella et al., 2008). In general, since the rows of F_1F_0-ATP synthase dimers are at the apex of the cristae, they could force a strong local curvature and consequently they shape the cristae. Nevertheless, the precise role of ATP synthase in regulating curvature of cristae is still a matter of debate: in particular it is still uncertain whether the mitochondrial anomalies observed when ATPase assembly is impaired are direct. For example, Bornhövd and colleagues put forward the hypothesis that impaired formation of F_1F_0-ATP synthase supracomplexes leads to an overall increase in plasticity or fluidity of the inner mitochondrial membrane, disruption of lipid microdomains, reduced flux through the respiratory chain that result in a lower membrane potential, without directly affecting cristae morphology (Bornhovd et al., 2006).

3.5.5. OPA1

Optic Atrophy 1 (OPA1), the homologue of *S.cerevisiae* Mgm1p, is the only dynamin-related protein identified in the inner membrane so far (Olichon et al., 2002). Since in this thesis we will focus on the OPA1 and its important role in the morphology and architecture of mitochondrial cristae, here we will discuss in detail the actual knowledge on OPA1.

ATP synthase may regulate positive curvature of IMM but it is still unclear how the negative curvature nearby cristae junction can be achieved and modulated in time. Dynamin related proteins can be natural candidates for this and mgm1/OPA1 is the only one so far known to be resident in the IMM. EM analysis of Mgm1/OPA1-depleted cells clearly demonstrated that this protein may have a role in cristae maintenance since disorganized cristae with irregular shape, some of which with large cristae junctions were observed (Olichon et al., 2003; Griparic et al., 2004; Sesaki et al., 2003). A possible role of OPA1/Mgm1 in structuring cristae is consistent with its cristae localization, as confirmed by

biochemical (Griparic et al., 2004; Olichon et al., 2003; Pelloquin et al., 1999; Wong et al., 2000) and immunogold staining (Vogel et al., 2006; Vogel et al., 2006). Interestingly, Amutha et al. have reported that Mgm1p is required for oligomerization of ATP synthase (Amutha et al., 2004); this result causally associates the cristae derangement typical of OPA1 depleted cells to an aberrant oligomerization of ATP synthase rather than a establishing a direct effect of OPA1 on cristae morphology. In 2006, our laboratory. demonstrated that *Opa1* depleted cells show disorganized cristae and they are more prone to apoptosis. Interestingly OPA1 can regulate cytochrome *c* mobilization and apoptotic cristae remodeling independently of its pro-fusion activity; moreover, OPA1 can regulate cristae morphology organizing into high molecular weight complexes that are target by BID during apoptosis. This correlates with the remodellling of the cristae that occurs during apoptosis (Frezza et al., 2006). The group of Nunnari proposed a similar model in yeast where Mgm1 was found to be required to tether and fuse mitochondrial inner membranes. Using specific fusion assay, they observed an additional role of Mgm1 in inner-membrane dynamics, specifically in the maintenance of cristae structures trough Mgm1 interactions on opposing inner membranes and proposed a model for how the mitochondrial dynamins function to facilitate fusion (Meeusen et al., 2006). From these data we can conclude that OPA1 has a critical role in controlling apoptotic cristae remodeling but the exact beyond this is still elusive and under investigation in our and many other groups.

Gene structure

The human *OPA1* gene is composed of 30 coding exons distributed across more than 90 kb of genomic DNA on chromosome 3q28-q29. *OPA1* is expressed ubiquitously with the highest levels in retina, brain, testis, heart and muscle (Alexander et al., 2000). Alternative splicing of exons 4, 4b and 5b leads to differentially expressed isoforms with open reading frames for polypeptides of 960–1015 amino acids (Delettre et al., 2001). The protein contains a mitochondrial leader sequence within the highly basic N-terminal targeting the protein to the outer surface of the mitochondrial inner membrane; a GTPase domain, a central dynamin domain that is conserved across all dynamins; and a carboxy-terminus coiled-coil domain responsible of protein-protein interactions. Its splicing region corresponds to a part of the protein with unknown function located between the mitochondrial leader sequence and the GTPase domain.

Protein structure

A further and perhaps even more crucial level of control is exerted by the proteolytic cleavage of Mgm1/OPA1. Mgm1p can be cleaved by the rhomboid proteases Pcp1p (Sesaki et al., 2003; McQuibban et al., 2003; Herlan et al., 2003) to produce a short (s-), soluble and a long (l-), membrane anchored form of Mgm1p with different function. The regulation of OPA1 processing in mammalian cells is matter of intense studies: this field is complicated by the fact that in mammalian cells OPA1 is present in 8 isoforms (Delettre et al., 2001) that are the result of alternative splicing and proteolytic processes at two sites: S1 and S2. Interestingly Ishihara and colleagues found that different isoforms of long form (-L) of OPA1 can be processed into two different short forms, according to the exons present, by paraplegin, a matrix facing AAA protease (m-AAA). Dissipation of membrane potential, expression of paraplegin, or induction of apoptosis stimulated this processing along with the mitochondrial fragmentation; Together, these results confirmed that the L-isoforms are the fusion-active species of OPA1 (Ishihara et al., 2006).

The group of Reichert confirmed that mitochondrial depolarization induces OPA1 cleavage that causes fragmentation of mitochondrial network; the fragmentation that occurs by a block of mitochondrial fusion depends on the normal DRP1-dependent fission pathway. However, they showed that processing of OPA1 appears to occur independently from ongoing mitochondrial fission (Duvezin-Caubet et al., 2006). Our lab demonstrated that the rhomboid protease PARL, the mammalian orthologue of Pcp1p, can participate in the production of a relatively scarce soluble, IMS form of OPA1. This soluble OPA1 has a role in apoptosis but not in the regulation of mitochondrial morphology (Frezza et al., 2006; Cipolat et al., 2006).

Duvezin-Caubet and colleagues in 2007 demonstrated that the protease involved in the $\Delta\psi_m$-dependent processing in not paraplegin itself (Duvezin-Caubet et al., 2006; Ishihara et al., 2006) but a component of the m-AAA protease complex, namely AFG3L2. Using reconstituted OPA1 processing in yeast to examine the involvement of the rhomboid protease PARL and the paraplegin-containing *m*-AAA protease, a mass spectrometric characterization of OPA1 isoforms not only revealed their formation by alternative splicing and proteolytic processing in HeLa cells but also that OPA1 is recognized and cleaved by *m*-AAA protease isozymes complexes composed of murine Afg3l1, Afg3l2, or human AFG3L2 subunits, but not by PARL (Duvezin-Caubet et al., 2007). In addition it was shown that a AAA protease acting in the intermembrane space (i-AAA) can process OPA1 in a

$\Delta\psi_m$-independent fashion: isoforms of OPA1 containing exon 4b and 5b seems to present an additional cleavage site for the i-AAA Yme1L; in addition, combination of long and Yme1L-processed OPA1 isoforms is important for mitochondrial fusion activity (Song et al., 2007; Griparic et al., 2007). A similar pattern of processing was confirmed by another group (Guillery et al., 2007). Interestingly, Baricault and colleagues demonstrated that apoptosis induction and PTP opening, as well as $\Delta\psi_m$ dissipation induce OPA1 cleavage. Decreased mitochondrial ATP levels, generated by apoptosis induction, depolarization or inhibition of ATP synthase, is the common and crucial stimulus that controls OPA1 processing (Baricault et al., 2007).

Recently, a paper from the group of Langer (Ehses et al., 2009) deepened our knowledge of the role of the m-AAA subunits AFG3L1 and 2. Concomitant loss of AFG3L1 and 2 in MEFs causes the accumulation of the S-form of OPA1 and leads to mitochondrial fragmentation, independent from Drp1. Thus, the L-form of OPA1 is clearly required for its fusion activity. Accumulation of short OPA1 in MEFs deficient for AFG3L1 and 2 is due to an enhanced cleavage of S1 triggered by the downregulation of m-AAA subunits. In order to identify what was the molecular mechanism, they looked for a protease that could mediate the processing and the degradation of the L-form of OPA1 in m-AAA deficient cells. They identified this novel protease in OMA1, previously described as metalloprotease whose activity overlaps with that of the m-AAA protease *(Kaser et al., 2003).* The depletion of OMA1 in AFG3L1 and 2 deficient cells stabilizes the L-form of OPA1 and rescues the morphology of the mitochondrial network. Further, they demonstrated OMA1 *per se* does not affect the cleavage of OPA1, but it is activated upon mitochondrial potential dissipation, loss of mtDNA, and apoptosis.

In conclusion, the pattern of cleavage of OPA1 is starting to be unraveled and it appears that the iAAA proteases are required for the stability of the long form, which is conversely cleaved by OMA1 in dysfunctional mitochondria. Parl, on the other hand, has an accessory role cooperating in the production of a soluble form of OPA1 that participates in the regulation of cristae shape especially during apoptosis.

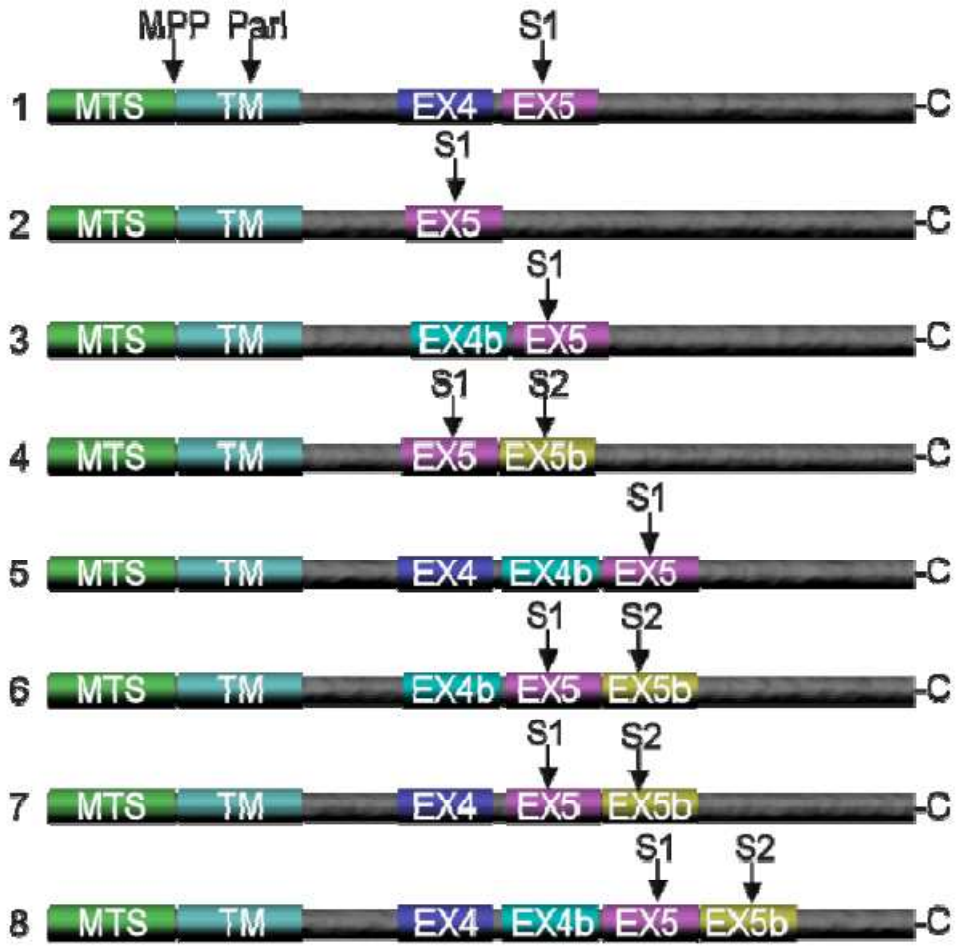

Fig. 4 Schematic of the eight OPA1 mRNA splice forms. The mRNA splice forms differ in the presence or absence of exon 4,4b and 5b. Cleavage of the mitochondrial targeting sequence (MTS) by MPP leads to the long isoforms. Additional cleavage at site S1 (exon5) or S2 (exon 5b) leads to the short isoform. TM, transmembrane domain

3.6. Genetic diseases of mitochondrial-shaping proteins

3.6.1. ADOA

Heterozygous mutations of *OPA1* cause autosomal dominant optic atrophy (ADOA), the most common form of inherited optic neuropathy, with an estimated prevalence of 1:50000 (Alexander et al., 2000; Delettre et al., 2000). Linkage analysis has revealed that *OPA1*, mapping to 3q28-q29, is the major locus (Trimmer et al., 2000; Votruba et al., 1997). A positional cloning approach identified this gene as being responsible for OPA1-type DOA (Alexander et al., 2000). ADOA is a specific disease of the retina characterized by retinal ganglion cells (RGC) degeneration followed by ascending atrophy of the optic nerve. Very little is known on the pathogenesis of ADOA; the lack of pain and inflammation during the development of the disease suggest that apoptosis may play a key role in the loss of RGC. More than 117 different pathogenic mutations in Opa1 have been described (Ferre et al., 2005). The vast majority results in a truncated protein or affect the GTPase domain crucial

for the biological activity of dynamins. Since the disease is transmitted as a dominant trait, it has been suggested that these mutations either act as dominant negative or induce a condition of haploinsufficiency leading to the clinical phenotype. ADOA is characterized by decrease in visual acuity, tritanopia (dyschromatopsia characterized by confusion in the blue-yellow hues), sensitivity loss in the central visual fields, and pallor of the optic nerve (Votruba et al., 1998; Ferre et al., 2005). Classic ADOA usually begins before 10 years of age, with a large variability in the severity of clinical expression, which may range from non-penetrant unaffected cases up to very severe, early onset cases, even within the same family carrying the same molecular defect (Delettre et al., 2002; Carelli et al., 2004). Histopathology studies have shown diffuse atrophy of the ganglion cell layer that predominates in the central retina and loss of myelin and nerve tissue within the optic nerve (Kjer et al., 1996).

It remains unclear why Opa1-ADOA manifests with an apparently restricted clinical ocular phenotype, comprising RGC loss. Opa1 is ubiquitously expressed throughout the body: in the heart, skeletal muscle, liver, testis, and most abundantly in the brain and retina. In the human retina, Opa1 is present in the cells of the RGC layer, nerve fiber layer, the photoreceptor layer, and the inner and outer plexiform layers (IPL & OPL). RGC display high expression levels of Opa1 and a “threshold” effect can be an explanation for the specificity of the disease. How Opa1 mutations cause the clinical symptoms of ADOA remains to be clarified. Non-neuronal cells from patients with ADOA can have aggregated, fragmented or normal mitochondria (Delettre et al., 2000; Olichon et al., 2007); however, because data from only a few patients have been reported, it is not clear whether these findings are the norm. In addition, Opa1 mutations have been associated with reduced ATP production and reduced mtDNA content (Lodi et al., 2004; Kim et al., 2005; Lodi et al., 2004). The defects that have been documented in human ADOA diseased tissue are not as severe as those observed in experimental cells in which OPA1 is depleted.

It has been also proposed an involvement of Opa1 in regulating the amount of mtDNA, as suggested by a study showing that ADOA patients may have slightly reduced mtDNA copy number in blood lymphocytes (Kim et al., 2005). Miss-sense point mutations in the highly conserved GTPase domain are responsible for a syndromic form of ADOA associated with sensori-neural deafness, ataxia, axonal sensory-motor polyneuropathy, chronic progressive external ophthalmoplegia and mitochondrial myopathy. These patients all harboured multiple deletions of mitochondrial DNA (mtDNA) in their skeletal muscle, thus revealing an unrecognized role of the Opa1 protein in mtDNA stability (Kim et al., 2005).

Mouse models of ADOA that contain truncated OPA1 develop some features of ADOA in an age-dependent manner (mati-Bonneau et al., 2007; Alavi et al., 2007). A small proportion of heterozygous mice show a progressive decline in retinal ganglion cell number and aberrations of axons in the optic nerve. Though variable in expression, the pathology in mutant mice eventually advances to a stage of nearly complete loss of RGCs and gliosis of the optic nerve that is very similar to histopathological studies in patients with severe ADOA. Ageing effects, as well as the effects of physiologically relevant stressors, such as light and intra-ocular pressure, may have important roles in the full manifestation of the phenotype. The mild phenotype of the heterozygous models, and the increased manifestation of anomalies with age, is therefore, not surprising. However, the relatively mild and variable phenotype could underline that haploinsufficiency is not the only mechanism responsible for the full blown phenotype and raise the question of whether a dominant-negative mechanism can be conversely responsible for the clinical manifestation of ADOA.

3.6.2. Charcot-Marie Tooth type IIA

In 2004, Zuchner et al. mapped the mutations responsible for Charcot-Marie-Tooth 2A (CMT2A), and identified *MFN2* as being the gene responsible for the disorder. CMT is one of the most common inherited disorders in humans, with an estimated prevalence of one in 2500 individuals. The clinical symptoms of CMT are distal weakness of the lower limbs, sensory loss, decreased reflexes and foot deformities. Other symptoms include cranial nerve involvement, scoliosis, vocal cord paresis and glaucoma. CMT neuropathies can be divided into 2 main groups, type 1 and type 2. In CMT1, nerve conduction velocities are considerably reduced. In CMT2, the nerve conduction velocities are normal but conduction amplitudes are decreased, due to the loss of nerve fibres (Zuchner and Vance, 2006). CMT2A is a neurodegenerative disorder characterized by the loss of sensory and motor axons at early stages of the disease, resulting in the degeneration of the neurons themselves during a later stage of the disease. The symptoms of CMT2A include moderate weakness and wasting of tibial muscles with lower limb hyporeflexia and mild distal sensory loss (Lawson et al., 2005).

Mutations in *MFN2* account for approx. 20% of CMT2 cases, making this the most prevalent axonal form of CMT. Most *MFN2* mutations in CMT2A cluster within the GTPase and the p21RAS-binding domains and are missense mutations (Zuchner et al., 2004;

Lawson et al., 2005; Kijima et al., 2005). Recently, a *de novo* truncation mutation in *MFN2* has been associated to CMT2 and optic atrophy (also known as hereditary motor and sensory neuropathy VI, HMSN VI) (Zuchner et al., 2006). Two large clinical studies have studies the genetic mutation found in patients of CMT2A correlating them with the onset and the severity of disease but the molecular mechanism is still object of intense work. Recently it has been published that the lack of mitochondrial fusion in Mfn2 conditional knockout impairs dendritic outgrowth and spine formation (Chen et al., 2007). Moreover we demonstrated that Mfn2 plays an important role in tethering the ER to mitochondria modulating the Ca^{2+}signalling (de Brito and Scorrano, 2008). These works opened new perspectives in the study of pathogenesis of this disease. In conclusion, the existence of specific genetic diseases linked to components of the mitochondria-shaping machinery highlights the importance of the processes of fusion and fission for the life of the cell and ultimately for human health. As mentioned above, mitochondria-shaping proteins have been linked to a variety of sporadic, more prevalent diseases, but the association is not unequivocal and the mechanisms are likely to be extremely complex, requiring further investigation to be clarified.

3.7. The metabolic role of mitochondria

Pioneering biochemical studies have forged long ago the concept that the mitochondrion is the “energy powerhouse” of the cell. It is the centre of cellular energetic metabolism, being the principal source of ATP for eukaryotic cells: only 5% of ATP generated by glucose is provided by glycolysis.

The three major mitochondrial processes leading to ATP synthesis are:

- The tricarboxylic acid cycle, located in the mitochondrial matrix, in which NADH and $FADH_2$ are produced from organic compounds
- The mitochondrial electron transport chain (mtETC), in which electrons are sequentially transferred to oxygen by electron carriers, the respiratory chain complexes. To limit free energy dissipation, electrons from NADH are transferred stepwise from the IM-associated respiratory chain complexes with higher redox potential to the ones with lower. Complex I (NADH dehydrogenase) catalyzes the transfer of electrons from NADH to CoQ. Complex II (succinate dehydrogenase) transfers electrons directly from succinate to CoQ. Electrons are transferred by complex III (ubiquinone-cytochrome *c* reductase) from reduced CoQ to cytochrome

c, which in turns shuttle them to complex IV (cytochrome *c* oxidase). Complex IV finally catalyzes the electron transfer from cytochrome *c* to O_2. Electrons transfer in complexes I, III and IV is coupled to proton pumping from the matrix to the intermembrane space.

- The phosphorilating system, which uses the free energy supplied by the respiratory chain to catalyze the synthesis of ATP from ADP and P_i.

  The electron transport chain and the production of ATP by the phosphorylating system are collectively called oxidative phosphorylation (OXPHOS).

3.8. OXPHOS protein

3.8.1. Complex I: structure and function

Complex I (NADH dehydrogenase) is normally considered as the "entry point" of electrons to the respiratory chain. It catalyzes the transfer of electrons from NADH to ubiquinone using a cofactor (FMN), eight redox groups and a iron-sulfur group. Complex I is the biggest complex of the OXPHOS, formed by 45 subunits codified by mtDNA and nuclear DNA. Until now, its crystal structure has not been obtained but by electron microscopy it has been showed that it has a "L" shape, with two perpendicular harms (Sazanov et al., 2000).

Since complex I is formed by many subunits, its assembly has not been fully characterized. The first assembly model was published by the group of Shoubridge (Antonicka et al., 2003). Blue Native Page-SDS bidimensional gels samples of patients carrying mutations on different subunits of complex I indicated that the assembly of the two arms takes place on the membrane by the sequential assembly of the different subunits. Many other studies further defined and characterized the assembly of complex I (Ugalde et al., 2004; Vogel et al., 2007; Dieteren et al., 2008). The actual state of knowledge supports the hypothesis that there is a first module of two subunits (NDUFS2 and NDUFS3) that is anchored to the mitochondrial membrane by ND1 and functions as a core for the assembly of all the other subunits.

3.8.2. Complex II: structure and protein

Complex II or succinate deydrogenase is formed by four subunits all codified by the nuclear genome, three iron-sulfur groups (Fe-S), a molecule of FAD and a group of cytochrome *b*. Complex II has two functions: during the Krebs cycle it transforms succinate into fumarate and during the electron transport chain it transfers electrons from

$FADH_2$ to ubiquinone. The structure of complex II is composed by two parts: one soluble that constitutes the catalytic units of the enzyme and one anchored to the membrane. The mechanism of assembly it is still unknown. Recently, Ghezzi and collegues described SDHAF1 as the first protein factor involved in the assembly of complex II (Ghezzi et al., 2009). Mutations in this protein lead to the failure of the assembly of the Fe-S cluster.

3.8.3. Complex III: structure and function

The complex III of the respiratory chain (ubiquinol:cytochrome *c* oxidoreductase or complex bc_1) catalyzes the transfer of electrons from ubiquinol to cytochrome *c* coupling this reaction with the pumping of four protons from the mitochondrial matrix to the intermembrane space. Complex III is a dimeric complex whose monomers are composed by 11 subunits, several redox groups and a dimer of Fe_2S_2. Only the cytochrome *b* subunit is codified by the mtDNA. The structural similarity of complex III between *S.cerevisiae* and mammals offered a great help in the studies on its assembly. Recently the assembly of complex III was discovered to be a sequential process which start from the assembly of two subcomplexes of cytochrome *b*, Qcr7p and Qcr8p (Zara et al., 2009).

3.8.4. Complex IV: structure and function

Complex IV or cytochrome *c* reductase is the last complex of the electron transport chain. It catalyzes the oxidation of cytochrome c donating the electrons to O_2 to generate H_2O. This reaction is coupled by the pumping of two protons. Complex IV in mammals is composed by 13 proteins. Three subunits are codified by the mtDNA and they are functional characterized: COI which possesses the heme groups, COII with a central copper group important for the bond with cytochrome *c* and COIII that is important for proton pumping. The nuclear encoded subunits are not functionally characterized but it seems that they are involved in the assembly of the complex. The dimerization is important for its activity and the assembly of the fully dimeric complex occurs by means of three subcomplexes (Fernandez-Vizarra et al., 2009).

3.8.5. ATP synthase: structure and function

ATPsynthase (also called complex V) catalyzes the synthesis of ATP from ADP+P_i using the energy of the electrochemical proton gradient generated by the electron transport chain. The complex is composed by 16 proteins that form two functional domains: F_1 and F_O (Futai et al., 1989). The F_1 region is localized in the mitochondrial matrix, is composed by 5 subunits and contains the catalytic domain of the protein. The F_O fraction is integral to

the membrane and acts as a proton pore transferring protons to the F_1 by a rotatory movement (Stock et al., 1999). When the membrane potential decreases, the ATP synthase could also hydrolyze ATP to ADP and functions as ATPase. This functional switch is controlled by the IF1 subunits (Cabezon et al., 2003).

The first step of the assembly of the ATP synthase is the formation of F_1, followed by the formation of an intermediate complex called V* on which the assembly of all the other subunits takes place (Nijtmans et al., 1995). Mutants of the mtDNA encoded subunits fail to assemble the ATPsynthase; they show intermediates of lower molecular weight. The same happens when the mitochondrial protein synthesis is impaired (Houstek et al., 1999) and in ρ^0 cells (Carrozzo et al., 2006).

3.8.6. Supercomplexes of respiratory chain (RCS)

The organization of the complexes of electron transport chain has been demonstrated to be very complicate. Many model of organization have been put forward and recent discoveries are giving us a more precise picture of the functional organization of complexes into supercomplexes. The first model was hypothesized by the group of Gupte after several years of experiments (Hackenbrock et al., 1986). This model was called “fluid model” an it was based on experimental data that confirm that all the complexes of the OXPHOS system can be purified as single units preserving their enzymatic activity. This model describes the complexes as single units which freely diffuse in the mitochondrial inner membrane and the electron transport between them occurs when the complexes meet each other by random collision. The “fluid model” was challenged by evidences that confirmed the existence of stable interactions between the complexes (a.k.a. the solid model) . The BlueNative page (BNGE) technique is an important tool for studying the protein complexes because it separates them preserving their physiological structure and their activity (Schagger, 1995). This technique gave an important improvement in the identification and characterization of the respiratory chain supercomplexes (RCS). Studies performed in yeast demonstrated that the respiratory chain is organized into functional units called supercomplexes (Boumans et al., 1998; Bianchi et al., 2004). Further studies, by taking advance of patient-derived cells, or of murine model of mtDNA mutation, revealed which complexes are important for the structure of supercomplexes. In particular, complexes III and IV are essential for the stabilization of complex I, and when they are mutated or not assembled, the assembly of supercomplexes is similarly impaired. Conversely, the absence of complex I affects supercomplexes formation but does not

impair the formation of the other complexes (Acin-Perez et al., 2004). Mass spectrometry showed that supercomplexes are defined by specific interactions between the complexes (Eubel et al., 2004; Schafer et al., 2006; (Heinemeyer et al., 2007). In particular, complex I and the always interacts with a dimer of complex III, while the association with complex IV seems to be dependent on the concentration and type of detergent used (Schagger and Pfeiffer, 2001). The supercomplexes are also functional units. Their activity has been measured by in activity assay in BNGE (Schagger and Pfeiffer, 2000b). Moreover, oxygen consumption rate of isolated plant mitochondria also correlated with the amount of RCS (Eubel et al., 2004). Recently, several groups demonstrated that supercomplexes are important structure for the cellular respiration in mammalian systems. Experiments in hybridomas demonstrated that the formation of supercomplexes sets the threshold for the complementation of mutations in complex III and complex IV (D'Aurelio et al., 2006). The existence of the supercomplexes has been fiercely opposed by researchers claiming that they are an artifact of the isolation by blue native gel electrophoresis. Recently, a biochemical approach showed that supercomplexes are functional protein complexes, with their own measurable respiratory activity, and that their assembly is consequent to the assembly of the single complexes as indicated by pulse-chase assembly assays of radiolabeled mtDNA encoded proteins (Acin-Perez et al., 2008). Thus, electron transport needs close association between the complexes in order to be efficient. Based on this work, a new "*plasticity model"* which integrates the two previous models has been proposed. According to the plasticity model, the complexes of respiratory chain are single protein units that are assembled into functional structures that are not fixed, but can change to support the different energy needs of the cells. However, the molecular mechanism and the regulatory factor of the assembly of RCS are still unknown. In particular, it is unclear if specific factors are required to assemble them or whether this assembly process relies solely on the availability of large membrane structures. This thesis investigate the effects of cristae remodeling focusing in the role of OPA1 in the assembly of RCS.

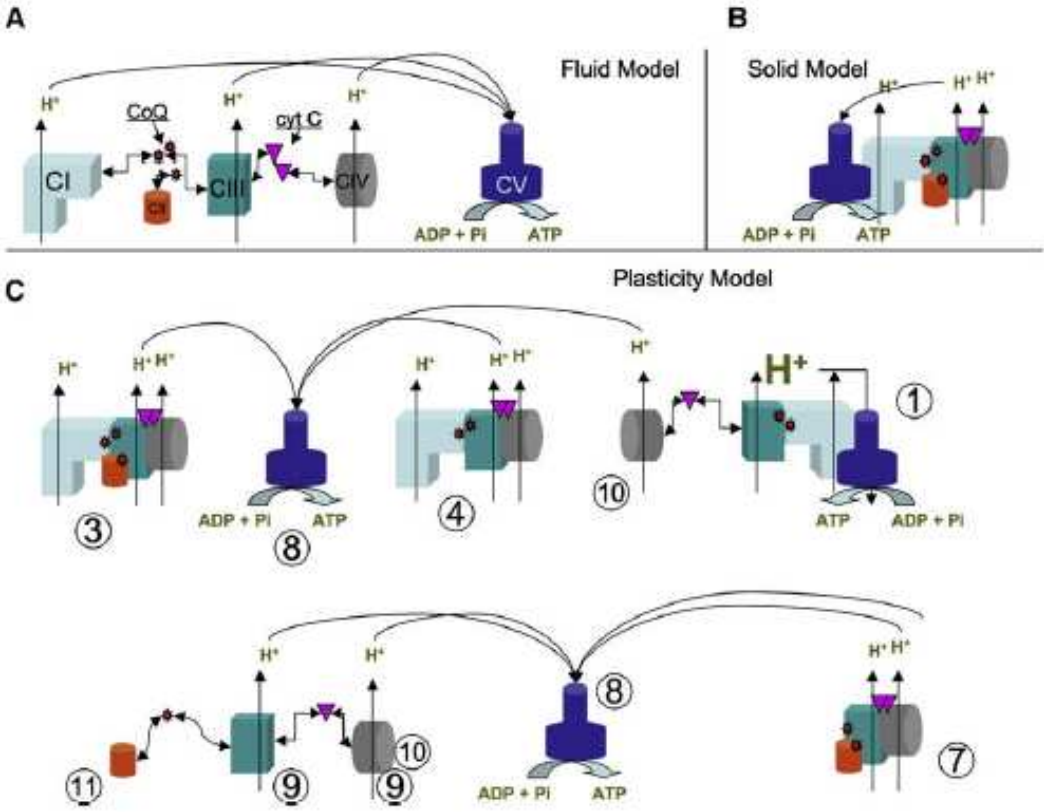

Fig.5 Schematic representation of the "fluid model" (A) , "solid model" (B) and the new "plasticity model" for the organization of OXPHOS complexes. The shape and colours code for indicating the complexes is descrived in (A). Coenzyme Q is represented as a small red-filled stars and cytochrome *c* as violet- filled triangles. From (Acin-Perez et al., 2008).

3.9. Apoptosis

Programmed cell death (Lockshin and Williams, 1965) and its morphologic manifestation called apoptosis is a conserved pathway that in its basic tenets appears operative in all metazoans. Cell deaths during embryonic development are essential for successful organogenesis and for crafting complex multicellular tissues. The evolutionary advent of differentiated cell types may have asked for a control over cell death as well death as division, in order to keep neighboring cells interdependent and to insure the proper balance of each cell lineage. Apoptosis also operates in adult organisms to maintain normal cellular homeostasis. This is especially critical in long-lived mammals that must integrate multiple physiological as well as pathological death signals, for example includes regulating the response to infectious agents. Gain- and loss-of-function models in the core apoptotic pathway indicate that interference with these pathways can be a primary disease-inducing pathogenic event (Danial and Korsmeyer, 2004). There are two main

pathways of apoptosis in mammals: the intrinsic and the extrinsic pathway of apoptosis. They differ for the type of trigger stimuli and for the relative role of mitochondria (Scaffidi et al., 1999; Scaffidi et al., 1998). The intrinsic pathway is initiated by an internal cell damage such as DNA damage, hypoxia or lack of nutrients that trigger the release of cytochrome *c* from mitochondria. The extrinsic pathway is triggered by the activation of "death receptors" that after the formation of the protein complex called "death inducing signaling complex" (DISC) lead to the activation of caspases-8 followed by the activation of the effector caspases-3. In certain cells type like hepatocytes the level of DISC is not sufficient to directly activate caspases-8, so that it requires a mitochondrial amplification loop. The main player of this amplification loop is the BH3-only member of the Bcl-2 family protein, BID (Danial and Korsmeyer, 2004). Therefore, mitochondria are not innocent bystander: the existence of a mitochondrial pathway of apoptosis substantiates the earlier discovery by Korsmeyer and coworkers that the antiapoptotic oncogene BCL-2 targets its product to mitochondria (Hockenbery et al., 1990). Indeed, after the activation of both pathways, mitochondria release in the cytosol cytochrome c that in complex with Apaf-1 activate caspase 9 which in turn activates the effector caspase 3.

The mitochondrial pathway of cell death is finely regulated by the BCl2-family proteins. These proteins, according to their BH domains, are classified inanti or pro-apoptotic members. The BCL-2 family members possess conserved α-helices with sequence conservation clustered in BCL-2 homology (BH) domains. The antiapoptotic proteins like BCL-2, BCL-XL and Bcl-W exhibit the homology in all segments BH1 to 4. The proapoptotic molecules lack stringent sequence conservation of the first α-helical BH4 domain and can be further subdivided into ‘‘multidomain’’ and ‘‘BH3-only’’ proteins. Multidomain proapoptotic members such as BAX and BAK display sequence conservation in BH1-3 domains. BH3-only members like Bim, Bid, Bad, Bik, Noxa, Puma, Hrk, Bmf, display sequence conservation only in the amphipathic α-helical BH3 region (Scorrano and Korsmeyer, 2003). The BH3-only proteins are important players in communicating to mitochondria the death signals originating from both intrinsic and extrinsic pathways. The interaction between pro- and antiapoptotic proteins depends on the BH domains. The antiapoptotic proteins structurally have an hydrophobic grove formed by BH1,2 and 3 that is the site of interaction with the BH3 domain of the other members of the family. This interaction results in homo- or heterodimerization. BCL-XL interacts with the pro-apoptotic protein Bax inserting the BH3 domain in the hydrophobic grove of BCL-XL (Nouraini et al., 2000). However, proapoptotic proteins as BAX and BAK, not only interact with the BCL-2

antiapoptotic proteins, but also form oligomers in the mitochondrial membrane interacting with the BH3-only domain members (Desagher et al., 1999; Eskes et al., 2000). In turn, the BH3-only members are further defined functionally as sensitizer or activators. The sensitizer are Noxa. Bad and Bik and they exert their pro-apoptotic role interacting with the BCL-2 anti-apoptotic proteins. Whereas the activators (Bim, Bid, Puma) are able to activate directly BAX and BAK (Cheng et al., 2001; Ren et al., 2010).

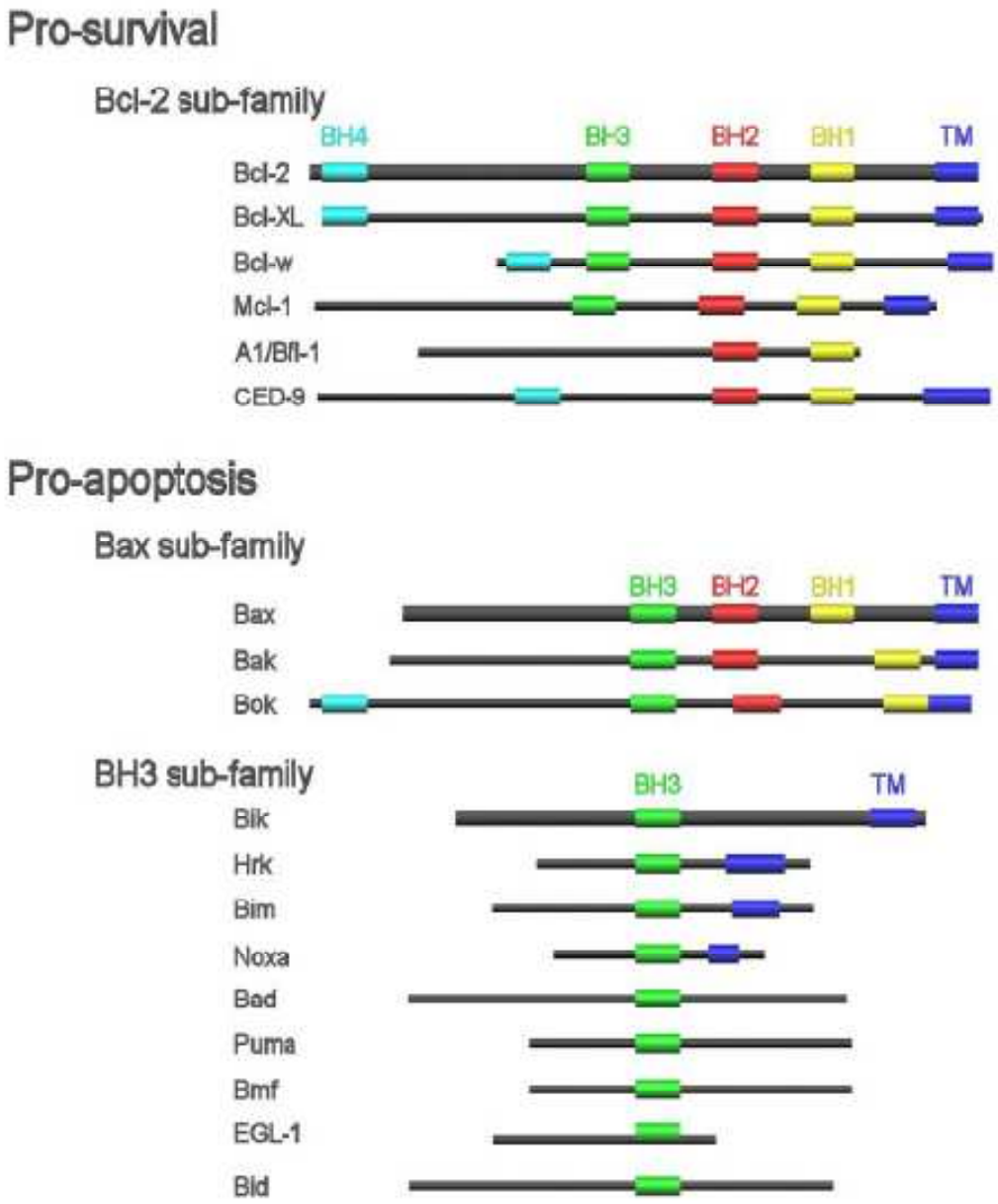

Fig.6 Summary of anti and proapoptotic BCL-2 family proteins. BCL-2 homology domains are highlighted

3.9.1. Mechanisms of cytochrome c release: OMM permeabilization

The key event for the cytochrome *c* release is the pemeabilization of the outer mitochondrial membrane. Oligomerization of the proapoptotic proteins BAX/BAK is required for the permeabilization of the mitochondrial outer membrane (Wei et al., 2000). A major question is how oligomerized multidomain BAX, BAK affect the release of proapoptotic activators from mitochondria. Current models include

(i) BAX, BAK oligomers generate a pore in the outer mitochondrial membrane permeable to cytochrome *c* and possibly to other proapoptotic proteins.

(ii) interactions between BAX and resident mitochondrial proteins such as VDAC (Shimizu et al., 1999; Roy et al., 2009b) or ANT (Marzo et al., 1998) which are proposed to release cytochrome *c* directly or trigger the mitochondrial permeability transition (PT);

(iii) a global effect on the permeability of the OM, this includes a number of possibilities, including a concept of ‘‘lipid’’ channels in the bilayer (Kluck et al., 1999; Kuwana et al., 2002).

As reviewed by Green and collegues (Chipuk et al., 2006) there are some evidences that MOMP occurs without direct decision made by mitochondria but is dictated by some interaction between cytoplasmic effectors and OMM: in this so called “innocent bystander scenario” the commitment to die or rather the commitment step that results in induction of MOMP rests entirely with the BCL-2 family of proteins and their regulators. The authors suggest that is not that the OMM or IMM does not participate in the induction of MOMP but that, if they do, they do so in a manner that always depends on the activities of the BH3-only or multidomain BCL-2 proteins in the cytosol. In this context, an injure acts first damaging mitochondria; the decision to undergo apoptosis, rather than necrosis, for instance, is made through some amplificatory loops that in the cytosol activate Bcl-2 members to promote MOMP. This scenario differs from classical view since Bcl-2 proapoptotic members are not seen as causative events of a damage to mitochondria, but rather as decision makers on promoting MOMP and hence apoptosis.

Irrespective of the exact mechanism by which active BAX, BAK release cytochrome *c*, it appears clear that these multidomain proapoptotics act as the essential gateway to the mitochondria, serving as the critical step of their engagement. Recent data indicate that

while this step is required, there are additional pathways downstream of the BH3-only proteins that ensure complete release of cytochrome *c*: they include remodeling of the cristae characterized by fusion of individual cristae and opening of the cristae junctions (Scorrano et al., 2002) and fragmentation of the mitochondrial network (Frank et al., 2001).

3.9.2. Beyond the OMM permeabilization: cristae remodelling

Any model of cytochrome *c* release must account for the rapid kinetics and complete extent of cytochrome *c* release (Goldstein et al., 2000). Discrete levels of cytochrome *c* are required in the cytosol to activate the death response (Zhivotovsky et al., 1998) and in certain cells the amount of cytochrome *c* released is critical to overcoming protection by the IAP caspase inhibitors (Deveraux et al., 1998). Since into the cristae are stored the major part of cytochrome *c,* it was hypothesized that complete release of cytochrome *c* must be due to a rearrangement of the mitochondrial ultrastructure. A turning point was the demonstration that following several death stimuli, including the BH3-only proteins BID (Scorrano et al., 2002) and BIK (Germain et al., 2005) or after Fas pathway activation (Mootha et al., 2001), mitochondria remodel their internal structure: individual cristae fuse and cristae junctions widen, to allow cytochrome *c* mobilization from its intra-cristae compartment toward the IMS for its subsequent release across the OMM according to the efflux pathway described in the previous section.

OPA1 is a key protein in the regulation of cristae remodeling (Frezza et al., 2006). OPA1 forms high molecular weight oligomers that comprise both the inner membrane integral form and the soluble intermembrane space one. These oligomers keep the cristae junction tight, like a clip between two adjacent inner membrane. During BID dependent apoptosis, the high molecular weight complexes of OPA1 are disassembled concomitantly to the opening of cristae junctions. The production of the soluble form of OPA1 requires the rhomboid protease Parl. In cells lacking Parl the oligomers of OPA1 are reduced and accordingly the cells are more sensitive to apoptosis. These data support a model where Parl and OPA1 act in the same pathway. Recently, the group of Newmeyer confirmed this model. Moreover they demonstrated that the cristae remodeling requires BAX but not its activation, confirming that cristae remodeling occurs before OMM permeabilization and that it is independent from it (Yamaguchi et al., 2008).

Many evidences underline the importance of the cristae remodeling in complete cytochrome *c* release. However many questions are still open on the molecular

mechanisms that trigger it. In particular it is still unknown what is the domain of BID required for cristae remodeling: the BH3 domain has been shown to be required (Yamaguchi et al., 2008) or not (Scorrano et al., 2002). In this thesis, we have address the role of the different domains of BID in cristae remodeling and we identified it in the α6-helix domain, which has also been implied in the binding of BID to lipids such as cardiolipin that have also been involved in the regulation of cristae shape during apoptosis (Epand et al., 2002). Recently it has been reported that the BH3-only protein Bnip3, which is induced by hypoxia, promotes mitochondrial fragmentation leading to apoptosis by interacting with OPA1 in vitro and in vivo that Bnip3 (Landes et al., 2010). This interaction is due to the carboxy-terminal transmembrane domain of Bnip3 that integrates into the OMM and leaves the last 10 residues into the intermembrane space where they can contact OPA1. This mechanism causes the destabilization of the high molecular weight complexes of OPA1. These data encourage the idea that also BID could interact with OPA1 or a protein incorporate in the high molecular weight complexes of OPA1. Further questions remain on the effects of cristae remodeling on mitochondria physiology, which have also been addressed during this thesis .

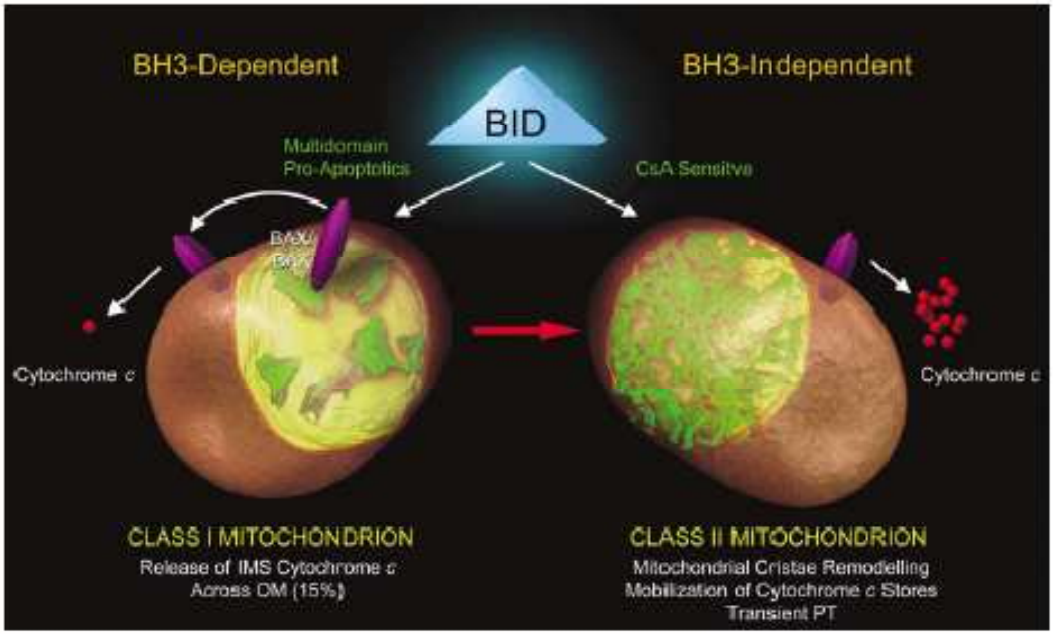

Fig. 7 Cartoon depicting the two apoptotic pathways triggered to reach complete cytochrome *c* release. Following an extrinsic apoptotic stimulus, BAX and BAK oligomerize allowing the cytochrome *c* release of the initial 15% stored into the intermembrane space. The BH3 independent pathway induces inner membrane remodelling and results in the mobilization of the 85% of cytochrome *c* stored in the cristae. From (Scorrano and Korsmeyer, 2003).

3.9.3. Beyond the OMM: mitochondrial fragmentation

A growing body of evidence suggests that mitochondrial- shaping proteins participate in cell death. Dnm1p, the yeast orthologue of DRP-1, mediates mitochondrial fragmentation and apoptosis-like death in S. cerevisiae (Fannjiang et al., 2004). Blocking Drp-1 in *C. elegans* inhibits apoptotic mitochondrial fragmentation and results in the accumulation of supernumerary cells during development (Jagasia et al., 2005). In mammalian cells, death by mitochondria utilizing intrinsic stimuli is accompanied by mitochondrial fragmentation and blunted by dominant negative DRP-1 (Frank et al., 2001). Similarly, expression of hFis1 results in cytochrome *c* release and death (James et al., 2003) and its downregulation by RNA interference prevents apoptosis to a greater extent than DRP-1 silencing (Lee et al., 2004). Alirol et al indicated that death by hFis1 relies on the ER gateway of apoptosis: hFis1 did not directly activate BAX and BAK, but induced Ca^{2+}-dependent mitochondrial dysfunction. Thus, hFis1 is a bifunctional protein that independently regulates mitochondrial fragmentation and ER-mediated apoptosis (Alirol et al., 2006). Of note, it was demonstrated by two separate groups that inhibiting DRP1-mediated mitochondrial fission by RNA interference (Estaquier and Arnoult, 2007; Parone et al., 2006) delayed but not prevented apoptosis and cell death: a likely explanation is that inhibition of Drp1-mediated mitochondrial fission partially prevents cytochrome *c* release but has no effect on the release of Smac/DIABLO, Omi/HtrA2, Adenilate Kinase and DDP/TIMM8a that can still mediate apoptosis. These data support the hypothesys that Drp1 has a relevant function in regulating cytochrome c egress trough OMM probably by impacting on ultrastructure of IMM.

Further analysis revealed that early in the course of cell death, MFN1 dependent mitochondrial fusion is largely inhibited (Karbowski et al., 2004) and combined overexpression of MFN1 and MFN2 protects from death by intrinsic stimuli like etoposide and BID (Sugioka et al., 2004). Loss of function of OPA1, in analogy to Mgm1p deficiency in yeast, leads to mitochondrial fragmentation. In vitro, RNAi-mediated knockdown of OPA1 causes extreme cellular sensitization toward exogenous apoptosis induction as well as spontaneous apoptotic cell death (Lee et al., 2004). In complex, these data seem to establish a linear correlation between fragmentation, blocking of fusion and apoptosis, but the picture is probably not so simple. First, not always fission promotes apoptosis, as confirmed by the ability of overexpressed DRP-1 to inhibit death by ceramide (Szabadkai et al., 2004). In this case, DRP-1 appears to protect by blunting the mitochondrial Ca^{2+} waves that transmit ceramide-mediated apoptotic signal (Pacher and Hajnoczky, 2001).

Inhibiting mitochondrial fission (even if we assume that complete knock out rather than silencing is obtained for each protein studied) does not lead to a complete inhibition of MOMP and cytochrome *c* release. This would be consistent with mitochondrial fission occurring after MOMP. Mitochondrial fission could be seen as a positive feedback to amplify MOMP and the release of cytochrome *c*. The amount of cytochrome *c* that is initially released, before recruitment of proteins of the fission machinery and mitochondrial fission, would be, in most cells, sufficient to trigger the formation of the apoptosome and caspase activation. Inhibiting mitochondrial fission would only slow down the kinetics of cell death.

The amount of cytochrome *c* that is required indicate that mitochondrial fission may be an amplificatory loop in apoptosis required to allow complete cytochrome *c* release. Intriguingly mitochondrial fission may also be a prerequisite for autophagy of the organelles and the connections between autophagy and apoptosis may also help illuminate how mitochondrial fission is coupled to the apoptosis cascade (Martinou and Youle, 2006).

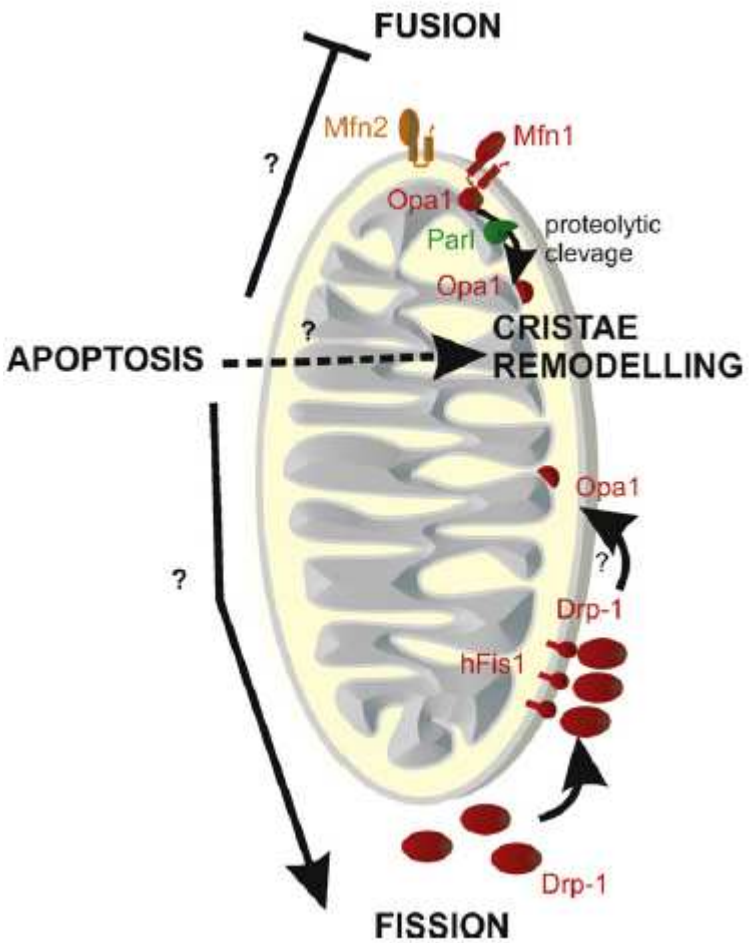

Fig. 8 Cartoon depicting the key events in mitochondrial morphology during apoptosis. The dynamin related proteins involved in the processes are reported. From (Scorrano, 2009)

3.9.4. A focus on BID: "Research Highlight" (Cogliati and Scorrano, 2010)

Cell Research (2010) 20:863-865.

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RESEARCH HIGHLIGHT

A BID on mitochondria with MTCH2

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Apoptosis is a key process for tissue homeostasis and renewal. Its dysregulation is implicated in most human diseases, from cancer to neurodegeneration. Apoptosis is triggered by stimuli that damage the internal structures of the cell, or by specialized "death" receptors on its surface. In certain cell types, Bid, a "BH3-only" member of the Bcl-2 family of death regulators integrates these two pathways at the mitochondrial level. Despite years of intense research, the mechanisms by which Bid translocates to mitochondria remain unclear. A recent study by Gross and colleagues sheds new light on this process [1]. They identified MTCH2 as a mitochondrial protein that interacts with Bid and whose ablation dramatically affects mitochondrial translocation of this BH3-only protein. Interestingly, MTCH2 shares homology with members of the mitochondrial carrier family, but it is located on the outer membrane of the organelle; and it was recently reported to be associated with increased body mass index. Thus, this study not only unveils how BID is targeted to mitochondria during apoptosis, but also opens interesting avenues to investigate the relationship between mitochondria, apoptosis and control of metabolism.

Programmed cell death or apoptosis is a conserved pathway in all metazoans. It is fundamental in embryonic development, organogenesis and in maintaining tissue homeostasis in adult organisms. Impairment of apoptotic pathways leads to cancer, while their upregulation results in degenerative disease. In mammalian cells, there are two main pathways downstream of death signals that are linked in certain cell types: the "death receptor" pathway triggered by extrinsic stimuli (e.g. Fas, TNFα) and the mitochondrial pathway triggered by intrinsic death stimuli (e.g. DNA damage). Both culminate in the activation of caspases, cysteine proteases that cleave a number of substrates involved in maintenance of cytoskeletal and nuclear integrity, cell cycle progression and DNA repair, resulting in the orderly demise of the cell. Mitochondria participate in the competent activation of caspases, by releasing cytochrome *c* and additional apoptogenic factors from the intermembrane space into the cytosol. Cytochrome *c* in complex with Apaf-1 activates caspase 9 and other downstream "effector" caspases. The key regulators of this apoptotic process are proteins of the Bcl-2 family which orchestrate the signals leading to the activation of effector caspases [2]. In response to the activation of death receptors, the apical pro-caspase 8 undergoes autoproteolytic activation. In type I cells, such as thymocytes, active caspase 8 directly cleaves the effector caspases 3 and 7, whereas in type II cells, such as hepatocytes, the activation of the effector caspases requires the mitochondrial amplification loop. To this end pro-caspase 8 is recruited on mitochondria where it binds to cardiolipin [3]. There, after self activation induced by proximity, it cleaves the proapoptotic BH3-only member, BID, and activate it. The active form, christened truncated BID (tBID) on the surface of mitochondria, triggers the release of cytochrome *c* by causing oligomerization of BAX and BAK that results in outer membrane permeabilization [2] and by inducing Opa1-dependent cristae remodelling [4-6]. The importance of tBID in the Fas death pathway is well established, however the molecular mechanism of tBID recruitment on mitochondria is still unknown and this has been an area of intense investigation in the last years. Two main models have been put forward to explain the affinity of BID for mitochondria: one postulates that BID travels to mitochondria as a consequence of its affinity for specific lipids, or of its specific lipidation; the other involves the existence of one or more specific receptors on the mitochondrial surface that interact with tBID to assist its insertion in the mitochondrial membrane.

The first model is supported by earlier studies that indicated how N-terminal myristoylation increases the affinity of tBID for the organelle [7]. In addition, tBID binds to the phospholipid cardiolipin (retrieved only in mitochondria), which proved to be required for tBID

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action [8]. Interestingly, cardiolipin on the outer mitochondrial membrane can also function as a scaffold for caspase 8, which translocates to mitochondria where it produces locales of BID [3]. In addition, the lipid composition of liposomes crucially modulated the ability of BID to permeabilize them, further substantiating a role for lipids in the action (and the targeting) of tBID [9]. In the second model, biochemical studies have substantiated a role for several mitochondrial proteins as receptors for tBID recruitment [10]. These include VDAC, as well as components of the mitochondrial protein import machinery, like TOM20, 22, 70 and 40 (reviewed in [11]), however, conclusive evidence for their role in this process is often lacking.

In a recent paper, Zaltsman *et al.* provide new important insights into the mechanism of recruitment of tBID on mitochondria. They define MTCH2/MIMP as a receptor for tBID on mitochondria and establish, using animal models, the importance of MTCH2/MIMP protein in Fas-induced hepatocellular apoptosis [1]. MTCH2/MIMP is a protein of the mitochondrial carrier family. In TNF-α treated cells, it resides in a 185 kDa large complex that comprises also tBID and BAX [12]. This evidence raised the hypothesis that MTCH2/MIMP could be involved in the mitochondrial apoptotic program but its role was not clear. Now, the paper of Zaltsman *et al.* closes this gap.

The first outstanding question that Zaltsman *et al.* tackled in their work was the submitochondrial localization of MTCH2. Being a member of the carrier superfamily, the natural prediction would be that it was located in the inner membrane. However, using three different biochemical approaches the authors clearly demonstrate that MTCH2/MIMP is in the outer membrane of the organelle. This result opened the possibility that MTCH2 participates in the steps of mitochondrial targeting of tBID during apoptosis. In order to verify this hypothesis, Zaltsman and coworkers assessed the role of MTCH2/MIMP *in vivo*. They first generated conventional knockouts, which were embryonically lethal when homozygous. They therefore created a conditional gene knockout mouse by using the Cre/loxP system. When MTCH2 is ablated, the cross-linkable complex tBID-MTCH2/MIMP is not detectable. Moreover, cells lacking MTCH2 are less sensitive to apoptosis induced by tBID and this defect is rescued by the reintroduction of MTCH2/MIMP. The reduced apoptosis is due to an impairment of tBID accumulation in mitochondria, following stimuli that converge on this BH3-only protein, including activation of Fas. Taken together, these results clearly demonstrated that MTCH2/MIMP protein is essential for the recruitment of tBID on mitochondria and it plays a fundamental role in the tBID-mediated cell death [1] (Figure 1).

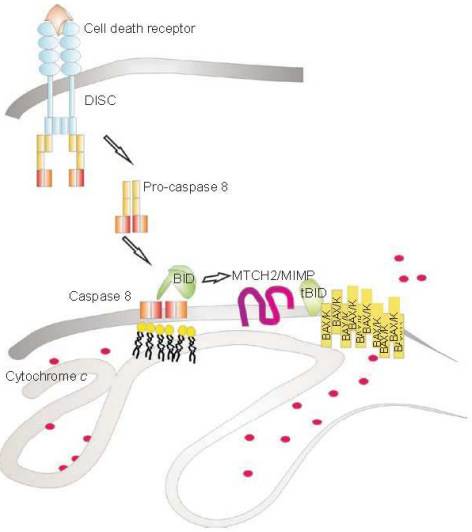

Figure 1 The recruitment of tBID on mitochondria is mediated by the novel target protein MTCH2/MIMP. The diagram depicts the sequence of events that occur in type II cells following an extrinsic death stimulus. Pro-caspase 8 binds to cardiolipin (yellow) on mitochondria where it undergoes self-proteolytic activation to cleave BID. The active tBID is then recruited on mitochondria by MTCH2/MIMP. This in turn leads to oligomerization of BAX/BAK and cytochrome *c* release.

It has been demonstrated *in vivo* that BID plays a key role in cell death induced by death-receptor ligands such as Fas ligand. In particular it exerts an important role in Fas ligand-induced apoptosis in hepatocytes [13]. Zaltsman and coworkers analyzed whether loss of MTCH2/MIMP that abolished *in vitro* recruitment of tBID on mitochondria could have significant effects on hepatocellular apoptosis *in vivo*.

They generated MTCH2/MIMP liver-specific knockout mice and assessed ther sensitivity to Fas. The liver-specific knockout animals show less liver injury and are more resistant to death than heterozygotes. In order to investigate the molecular mechanism of these effects, they analyzed the activation of caspases and the recruitment of tBID to mitochondria. The results clearly showed that in mice lacking MTCH2/MIMP in liver, caspase 8 was cleaved but the recruitment of tBID to mitochondria failed. This causes less activation of caspase 3 and consequently the hepatocites are less prone to apoptosis after Fas stimulation.

These results definitely confirmed that the MTCH2/MIMP protein plays a fundamental role in the recruitment of tBID to mitochondria and thus playing a key role in the Fas death pathway. Moreover, this paper contributes to clarify the basal mechanism of interaction of tBID with the mitochondria. Thanks to the paper of Zaltmans and co-workers, we now understand that the recruitment of tBID on mitochondria is at least partially orchestrated by a specific protein. However, it should be mentioned that the ablation of MTCH2 is not able *per se* to completely abrogate apoptosis, suggesting that other proteinaceous and/or lipidic receptors exist and play a role in the targeting of BID to mitochondria. Thus, it would be interesting to address if in cells lacking enzymes that participate in cardiolipin remodelling, like tafazzin [14], ablation of MTCH2 completely reduces it, substantiating a model in which lipids and proteins cooperate to target BID. Alternatively, one can envision a role for cardiolipin in targeting and/or assembly of MTCH2 in the outer membrane.

Another interesting link is that between MTCH2 and metabolism. MTCH2 recently emerged from a genetic screen as one of six new loci whose polymorphic variants are associated with increased body mass index. Among them, MTCH2 was the only one whose mRNA was not detected in the hypothalamus. This suggests that MTCH2 could play roles in the regulation of body mass in the periphery, as a regulator of energy expenditure. Apoptosis has been previously linked to metabolism when the BH3-only protein BAD was discovered to be a scaffold for enzymes of glucose metabolism on the surface of mitochondria, independently of its function in apoptosis [15]. However, in the case of MTCH2 it is not clear if the protein like BAD fulfils multiple functions in independent pathways, or if its role in apoptosis is key also for the regulation of body weight. In addition, it might be interesting to address if MTCH2 participates in the regulation of body weight by impacting on mitochondrial function. Studies capitalizing on the use of the conditional knockout animals generated by Zaltsman *et al.* will for sure help address these questions and place this protein in the broad context of integrated metabolism.

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4. Results

Opa1-dependent cristae remodeling disassembles respiratory chain supercomplexes, triggering apoptotic mitochondrial dysfunction

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OPA1-dependent cristae remodeling disassembles respiratory chain supercomplexes, triggering apoptotic mitochondrial dysfunction

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Summary

Remodeling of mitochondrial cristae, controlled by oligomers of Optic atrophy 1 (OPA1) mutated in dominant optic atrophy, supports the complete release of cytochrome *c* from mitochondria during apoptosis. Here we show that proper cristae shape is required for the assembly of respiratory chain supercomplexes (RCS), functional quaternary organizations of the respiratory chain complexes. Genetic dissociation of outer membrane permeabilization from remodeling of the cristae during cell death supports the pivotal role of the latter in apoptotic mitochondrial dysfunction. Ablation of *Opa1*, but not of the outer membrane pro-fusion proteins mitofusins, recapitulates the disassembly of RCS observed in apoptotic mitochondria. Genetic or apoptotic perturbation of cristae shape and of RCS assembly impairs the growth ability of cells relying on mitochondrial respiration. Thus, cristae shape is a key factor for assembly and function of RCS, determining mitochondrial dysfunction during apoptosis.

INTRODUCTION

Mitochondria are key organelles in intermediate cellular metabolism, energy production and calcium homeostasis (Dimmer and Scorrano, 2006). They also integrate and amplify apoptosis induced by several intrinsic stimuli by releasing cytochrome *c* and other proapoptotic factors required for the activation of caspases that orchestrate the orderly demise of the apoptotic cell (Green and Kroemer, 2004). Members of the BCL-2 family of proteins are key regulators of cytochrome *c* release and hence of apoptosis, by mediating the permeabilization of the outer membrane (OMM) to allow the release of the mitochondrial proapoptotic factors (Danial and Korsmeyer, 2004). Mitochondrial inner membrane (IMM) can be further divided in two distinct compartments, the so called "boundary membrane" and the cristae, separated from the former by narrow tubular junctions (Frey and Mannella, 2000). During apoptosis, shape of the cristae is altered: their curvature inverts and the tubular cristae junctions enlarge in a process that is required for the complete release of cytochrome *c*, normally confined in the cristae (Scorrano et al., 2002). Cristae remodeling is one of the two facets of mitochondrial shape changes occurring during apoptosis, being accompanied by a reversible fragmentation of the mitochondrial network that occurs around the time of cytochrome *c* release (Frank et al., 2001; Martinou et al., 1999).

A growing family of "mitochondria-shaping" proteins regulates fusion and fission events of mitochondrial membranes, ultimately affecting the morphology and ultrastructure of the organelle (Griparic and van der Bliek, 2001). Mitofusins (MFN) 1 and 2 are highly homologous of dynamin related proteins of the outer membrane and they orchestrate mitochondria fusion (Santel and Fuller, 2001; Legros et al., 2002; Chen et al., 2003; Santel et al., 2003). However, while the primarily role of MFN1 is to participate in mitochondria fusion cooperating with the dynamin-related protein Optic Atrophy 1 (OPA1) (Cipolat et al., 2004), MFN2 has an additional key role in ER-mitochondria tethering (de Brito and

Scorrano, 2008). Mitochondrial fission is regulated by the dynamin related protein 1 (DRP1) that is located into the cytoplasm and during fission translocates to mitochondria by calcineurin dependent dephosphorylation (Yoon et al., 2001; Smirnova et al., 2001; Cereghetti et al., 2008). Optic Atrophy 1 (OPA1), is the only dynamin-related protein identified in the inner membrane (IM) so far (Olichon et al., 2002). In addition to its role in mitochondria fusion, OPA1 has an antiapoptotic function which is genetically distinguishable from fusion (Frezza et al., 2006). OPA1 keeps the cristae junctions tight by forming oligomers that contain two forms of OPA1, one soluble in the IMS, generated by intramembrane proteolytic cleavage by the rhomboid protease PARL (Cipolat et al., 2006), and the second inserted in the IMM (Frezza et al., 2006). EM analysis and EM tomography shows that OPA1-depleted cells harbor disorganized cristae whose shape is irregular (Frezza et al., 2006). The cristae are key metabolic structures, since they are the site of oxidative phosphorylation where the complexes of respiratory chain are localized. Recent structural and functional evidence indicated that respiratory chain complexes are further organized into functional and dynamic quaternary supramolecular structures christened as supercomplexes (RCS), in order to improve the efficiency of electron channeling (Acin-Perez et al., 2008).

Remodeling of mitochondrial cristae was originally discovered to occur in response to the pro-apoptotic BH3-only BCL-2 family member BID, to require an unknown region of the protein beyond its BH3-domain, and to be independent of BAX and BAK and hence of outer membrane permeabilization (Scorrano et al., 2002). Further studies indicated that BID disrupts the OPA1-containing oligomers leading to cristae junctions opening; high levels of OPA1 stabilize them and prevent mobilization of cytochrome *c* from mitochondria (Frezza et al., 2006). Other BH3-only BCL-2 family members like BimS and Bnip3 were similarly found to disassemble OPA1 oligomers and to remodel the inner membrane of mitochondria (Yamaguchi et al., 2008; Landes et al., 2010). However, these recent studies

also raised questions as to the domains responsible for the remodeling of the cristae, which was confirmed to be separable from the activation of multidomain proapoptotics, but required the BH3 domain of BID (Yamaguchi et al., 2008).

While the role of cristae remodeling in the amplification of the apoptotic cascade has been investigated, its consequences on mitochondrial function are unknown. We therefore set to address if apoptotic cristae remodeling affects the activity and the structure of the mitochondrial respiratory chain supercomplexes (RCS), preferentially located in the cristae. Here we show that the shape of the cristae is fundamental for the correct assembly of RCS and hence for optimal mitochondrial respiratory function.

RESULTS

An α6-helix mutant of BID dissociates outer membrane permeabilization from cristae remodeling.

Outer membrane permeabilization and cristae remodeling occur simultaneously in apoptotic mitochondria, calling for a genetic tool to dissociate the two processes (Scorrano et al., 2002). A stretch of aminoacids in BID displays homology with Mastoparan (Fig. 1A), a 14-amino acid amphipatic wasp venom peptide that perturbs mitochondrial membranes (Pfeiffer et al., 1995) and is located in the hydrophobic α6-helix of the molecule (Fig. 1B). A similar sequence is found in the transmembrane domains of Bnip3 and BimS (Fig. S1A) that also remodel mitochondria and interfere with Opa1 (Yamaguchi et al., 2008; Landes et al., 2010). This stretch is highly conserved among metazoans, in particular in the two adjacent Lys residues 157 and 158 of *H. sapiens* BID (Fig. S1B). Since the hairpin of α6 and α7 is responsible for mitochondrial dysfunction elicited by BID (Gonzalvez et al., 2010), we reasoned that this region could be a good candidate to perturb cristae shape and mutagenized the two adjacent Lys (157 and 158) to Ala (BID^{KKAA}). A potential side effect of this mutation could be the reduced BID integration in the mitochondrial membrane, which also depends on the hairpin formed by the α6 and α7-helices (Wei et al., 2000). A carbonate extraction experiment conversely indicated that recombinant, purified caspase-8 cleaved $cBID^{KKAA}$ as well as an inactive BH3 mutant ($cBID^{G94E}$) and a double α6 and BH3 domain mutant ($cBID^{KKAAG94E}$) inserted into membranes of isolated mouse liver mitochondria (MLM) as efficiently as wt cBID (Fig. 1C). However, the KKAA mutant released cytochrome *c* less efficiently than wt, but more than the inactive G94E mutant of cBID (Fig. 1D) (Wei et al., 2000). Reduced release of cytochrome *c* could be caused by inefficient outer membrane permeabilization, sustained by oligomerization of BAX and BAK, or by impaired cristae remodeling (Scorrano et al., 2002). Furthermore, the interrelationship between cristae remodeling and outer membrane multidomain

proapoptotics is controversial: while originally the multidomains were found to be dispensable for the remodeling process (Scorrano et al., 2002), more recently their presence, but not their activation was deemed essential (Yamaguchi et al., 2008). Thus, we reasoned that the set of cBID mutants, together with genetic models of *Bax* and *Bak* ablation, could be a useful tool to clarify this issue. Crosslinking experiments showed that oligomerization of BAK was superimposable in mitochondria treated with cBID or cBID^{KKAA} (Fig. 1E), ruling out that this mutant is defective in outer membrane permeabilization. Conversely, when we turned to two established assays of cytochrome *c* mobilization from the cristae, we found that cBID^{KKAA} was considerably less efficient than wt cBID in increasing the ratio of ascorbate-driven over TMPD-driven respiration as well as the rate of cytochrome b_5 dependent extramitochondrial NADH oxidation (Fig. 1F,G), as opposed to the G94E mutant that we found earlier to be as efficient as wt in mobilizing cytochrome *c* (Scorrano et al., 2002). Finally, electron microscopy confirmed that cristae remodeling, as testified by the appearance of remodeled ("class II") mitochondria, is impaired in the cBID^{KKAA} mutant (arrowheads in Fig.1H). Thus, a mutant in the α6 helix of BID dissociates activation of BAX and BAK and hence outer membrane permeabilization, from remodeling of the cristae.

BID^{KKAA} is less efficient in disassembly of OPA1 oligomers and induction of apoptosis.

High molecular weight (HMW) oligomers of OPA1 are an early target of BID and their disruption is linked to the widening of the cristae junctions and to the mobilization of cytochrome *c* (Frezza et al., 2006). Chemical crosslinking and Blue Native gel electrophoresis (BN PAGE) showed they are disrupted by cBID (Fig. 2A,C and S2A). Interestingly, cBID^{KKAA} as well as the double cBID^{KKAAG94E} were less efficient than cBID or than its single G94E mutant in targeting OPA1 oligomers (Fig. 2 A,D and S2B). The

independence of the pathway of cristae remodeling from outer membrane permeabilization was further substantiated when we found that cBID could disrupt OPA1 oligomers also in mitochondria isolated from *Bax*, *Bak* doubly deficient (DKO) mouse embryonic fibroblasts (MEFs) (Fig. S3A), despite the lack of the release of the soluble fraction of OPA1 or of cytochrome *c* (Fig. S3B and not shown). Thus, the α6 mutant of BID is unable to induce OPA1-dependent cristae remodeling which occurs independently of the outer membrane multidomains BAX and BAK. We then wished to verify if hampering cristae remodeling had any effect on cell death. Expression of truncated BID (tBID) resulted in apoptosis of mouse embryonic fibroblasts (MEFs), as measured by the exposure of phophatidylserine on the outer leaflet of the plasma membrane, which was reduced, as expected, in the BH3 inactivating mutant. Interestingly, also $tBID^{KKAA}$ killed less than wt BID, supporting a role for cristae remodeling in apoptosis in situ. (Fig. 2E)

Disassembly of respiratory chain supercomplexes during cristae remodeling.

Cristae are the site of oxidative phosphorylation (Gilkerson et al., 2003) and BID destabilizes mitochondrial bioenergetics (Gonzalvez et al., 2005), thus recapitulating many of the changes that occur in mitochondria during intrinsic apoptosis. However, the relative contribution of outer membrane permeabilization vs. cristae remodeling is unclear and BID^{KKAA} can be a powerful tool to dissect them. Respiratory control ratio of purified mitochondria incubated with excess exogenous cytochrome *c* and NADH (to prevent changes due to IM or OM permeabilization) was reduced by cBID, only when mitochondria were energized with substrates for complex I (glutamate/malate), but not when they were feed with substrates entering the electron transport chain at complex II (succinate) or complex IV (ascorbate+TMPD) (Fig. 3A and S4) Interestingly, the BH3-only mutant $cBID^{G94E}$ that does not permeabilize the OM recapitulated these changes, while $cBID^{KKAA}$ or the double $cBID^{KKAA,G94E}$ mutant did not (Fig. 3B). These experiments suggested that

changes in mitochondrial bioenergetics during apoptosis by BID depend on complex I and are not caused by outer membrane permeabilization or caspases-feedback on mitochondria, but are specifically linked to the activation of the cristae remodeling.

Mitochondria energized with glutamate/malate transfer electrons from complex I to complex IV through complex III. These complexes of the respiratory chain are assembled into quaternary supercomplexes whose integrity is fundamental for respiration (Acin-Perez et al., 2008). Thus, in principle the cristae remodeling could impair RCR by interfering with the function of the RCS. In order to verify this hypothesis we turned to an in gel activity assay of complexes I and IV and of their supercomplexes, Interestingly, cBID but not cBID^{KKAA} reduced activity of RCS, leaving unaltered that of individual complexes I and IV (Fig. 3C and quantification in 3D) suggesting that cristae remodeling could alter RCS function. In principle, this impairment could be functional or structural, i.e. caused by changes in RCS supercomplexes during cristae remodeling. In addition, whether this is an in vitro artifact of the reconstituted system, or it occurs also in cells primed to apoptosis remained to be assessed. We therefore designed an experiment to simultaneously answer to these two questions. *Bax*, *Bak* doubly deficient (DKO) MEFs resistant to cytochrome *c* release were transduced with retroviruses encoding for tBID or tBID^{KKAA} and their 13 mtDNA-encoded proteins were radioactively pulse-chased to inspect assembly of RCS by BN PAGE (Acin-Perez et al., 2008). Autoradiography showed that in DKO mitochondria tBID, but not tBID^{KKAA} reduced the amount of radioactivity incorporated in the respirosome (I+III+IV) and to a lesser extent in the intermediate supercomplex I+III (Fig. 3E), resulting in a lower ratio of respirosome-assembled over monomeric complex I (Fig. 3F). Two-dimension BN/SDS-PAGE of radiolabeled mtDNA-encoded proteins from DKO MEFs transduced with tBID and the KKAA mutant allowed the separation of the respiratory chain subunits incorporated into complexes and supercomplexes, further elucidating that only wt BID destabilizes the structure of RCS (Fig. 3G). These experiments indicate that RCS are

destabilized in vitro and in vivo when BID can trigger cristae remodeling. Furthermore, they show that changes in RCS are independent of BAX and BAK and hence of outer mitochondrial membrane permeabilization or feedback mechanisms involving activated caspases.

Assembly of RCS is impaired in *Opa1*$^{-/-}$ cells

Is disorganization of RCS a general consequence of altered cristae shape? We turned to a genetic approach, capitalizing on *Opa1*$^{-/-}$ cells which have disorganized cristae (Frezza et al., 2006). BN PAGE followed by immunoblotting for subunits of the respiratory chain indicated lower levels of RCS in *Opa1*$^{-/-}$ mitochondria (Fig. 4A). Of note, the amount of monomeric complex I is also reduced, and an additional lower MW band appeared when BN PAGE of *Opa1*$^{-/-}$ mitochondria were immunoblotted for the subunit of complex I NDUF9. The important reduction in total complex I could explain the observed absence of RCS; however, blotting the BN PAGE for subunits of complex III and complex IV suggested that the reduction of the RCS was not the mere consequence of less monomeric complex I. Finally, reintroduction of OPA1 in *Opa1*$^{-/-}$ MEFs indicated that the defect was a specific consequence of *Opa1* deletion (Fig. 4A). To gain further insight into the mechanism of reduced RCS in *Opa1*$^{-/-}$ cells, we turned again to a two dimension BN/SDS-PAGE of radiolabeled mtDNA-encoded proteins from wt and *Opa1*$^{-/-}$ MEFs which revealed that not only *Opa1*$^{-/-}$ mitochondria contain less RCS, but also that their composition is aberrant (Fig. 4B). In *Opa1*$^{-/-}$ cells, less cytochrome *b* and CO1 (a complex IV subunit) were retrieved into the respirosome, as judged by the ratio of autoradiographic signals of these proteins between the respirosome and their respective monomeric complex (Fig. 4C). This data suggested that in *Opa1*$^{-/-}$ cells the mtDNA-encoded proteins remain in the monomeric complexes, which apparently fail to be assembled into supercomplexes, whose assembly is subsequent to the formation of single complexes in a

specific time dependent pattern (Acin-Perez et al., 2008). We therefore took advantage of a RCS assembly assay following over time the incorporation of radiolabeled mtDNA-encoded proteins into complexes and supercomplexes of the respiratory chain (Fig. 4D). When we compared the rate of radioactivity appearance in complexes and supercomplexes between wt and *Opa1*$^{-/-}$ mitochondria we noticed: (i) that the overall amount of radioactivity incorporated in individual complexes was reduced in *Opa1*$^{-/-}$ mitochondria as compared to wt. However, the relative rate of radioactivity incorporation in each individual complex (i.e. complex IV and V), is similar in wt and *Opa1*$^{-/-}$ mitochondria, indicating that the different steps leading to assembly of mtDNA-encoded subunits into complexes of the respiratory chain was not affected by ablation of OPA1 (Fig. S5A); (ii) that the ratio between radioactivity in RCS and that found in complex V was reduced throughout the chase period (Fig. S5B). Thus, assembly of RCS is delayed and even incomplete in *Opa1*$^{-/-}$ mitochondria. This is further confirmed by the lower incorporation of radioactivity in the *Opa1*$^{-/-}$ respirosome as compared to the intermediate, lower MW I+III supercomplex; (iii) that a smear of residual radioactivity remained below complex IV in *Opa1*$^{-/-}$ mitochondria, a signal of free, non incorporated protein subunits. These phenotypes were completely complemented by the reintroduction of OPA1(Fig. 4D), indicating that correct RCS assembly requires OPA1.

In addition to the perturbation of the RCS assembly, the overall radioactive signal of the mt-DNA encoded proteins was reduced in *Opa1*$^{-/-}$ mitochondria (Fig. 4B and S6), calling for an evaluation of the effect of *Opa1* ablation also on mtDNA levels. Real time PCR indicated an overall reduction of mtDNA copy number (Fig. S7A) a consequence of reduced mitochondrial fusion that plays a role in maintenance of mtDNA copy number (Chen et al., 2005). If the impaired RCS assembly observed in *Opa1*$^{-/-}$ mitochondria was consequence of reduced fusion and mtDNA copy number, it should be phenocopied by other genetic mutations that reduced mitochondrial fusion. MEFs lacking both *Mfn1* and *2*

are unable to fuse mitochondria (Chen et al., 2005) and as expected they displayed the same reduction in mtDNA copy number retrieved in *Opa1*$^{-/-}$ cells (Fig. S7B), while biogenesis of cristae was not affected as judged by a morphometric analysis (Fig. S8). A single BN PAGE as well as a 2D BN/SDS PAGE of radioactively labeled mtDNA-encoded proteins of *Mfn1*$^{-/-}$,*2*$^{-/-}$ mitochondria showed that even if a slight decrease in the overall radioactive signal proteins can be appreciated, RCS are correctly formed (Fig. 5A-B). This was further confirmed by a pulse-chase assembly assay that substantiated how lack of *Mfns* had no effect on the incorporation of individual components of the respiratory complexes into RCS (Fig. 5C). Taken together, these data indicate that *Opa1*$^{-/-}$ cells display a specific defect in RCS assembly that is not phenocopied in *Mfn1*$^{-/-}$,*2*$^{-/-}$ MEFs that share the same impairment in fusion and mtDNA copy number. Thus, shape of the cristae is likely to play a major role in RCS assembly.

Proper RCS assembly is required for efficient cell growth sustained by mitochondria.

Is there any physiological consequence of the rearrangement of RCS on cell metabolism? To address this question, we compared cellular growth in media rich in glucose or where this monosaccharide was substituted with galactose, which forces the utilization of the respiratory chain to produce ATP. Cells with defects in mitochondrial respiratory chain are unable to support ATP production via mitochondria. This is the case of the mouse A22 fibroblast, that do not synthesize cytochrome *b*, the only mtDNA-encoded protein of complex III, and grow normally only when glucose is present in the medium, dying when it is replaced by galactose (Acin-Perez et al., 2004) (Fig. 6A-B). Interestingly, *Opa1*$^{-/-}$ MEFs switched to galactose media grew slower, but they did not die like A22 cells. Since the total cell number results from the balance between cell growth and death, we excluded that *Opa1*$^{-/-}$ cells were dying more than their wt counterparts in galactose-containing media

(not shown). Finally, reintroduction of OPA1 confirmed that the growth defect in galactose was a specific consequence of *Opa1* ablation (Fig. 6B). Again, the slower growth when mitochondria are essential for ATP production could be a consequence of reduced mitochondrial fusion or of the lower mtDNA copy number. In this case it should be phenocopied by *Mfn1*$^{-/-}$*,2*$^{-/-}$ cells, which conversely do not display any growth defect in galactose (Fig. 6C,D), suggesting that changes in the shape of the cristae and hence assembly of the RCS are determinant to reduce metabolic efficiency of *Opa1*$^{-/-}$ cells.

Finally, we wished to extend these findings to apoptosis and to address if also cristae remodeling affected the ability of the cell to use mitochondrial metabolism to grow. However, triggering apoptosis results in outer membrane permeabilization and potential caspases-dependent feedbacks of mitochondrial dysfunction can be activated. We decided to turn to DKO cells where outer membrane permeabilization is blocked and transduced them with retroviruses coding for tBID. As expected, no changes in growth were observed when cells had access to excess glucose to support ATP production (Fig. 6E). Conversely, when DKO cells were switched to galactose, growth was impaired when cells were transduced with tBID and its BH3 mutant G94E, but not with the cristae remodeling deficient mutant KKAA (Fig. 6F). In conclusion, during apoptosis remodeling of the cristae impairs growth of cells dependent on mitochondrial metabolism, irrespective of outer membrane permeabilization.

Discussion

Here we demonstrate that proper shape of the cristae is required for assembly of the respiratory chain supercomplexes. Apoptotic as well as genetic perturbations in cristae morphology result in disassembly of the RCS and impair the respiratory efficiency of mitochondria, ultimately impacting on the growth of the cell when it depends on mitochondria for ATP generation.

Considerable controversy exist as to the mechanism and the importance of cristae remodeling in the release of cytochrome *c* during apoptosis. Changes in mitochondrial ultrastructure have been however reported in several settings and have been unequivocally correlated to a faster release of cytochrome *c* from the organelle (Scorrano et al., 2002; Germain et al., 2005; Frezza et al., 2006; Yamaguchi et al., 2008; Merkwirth et al., 2008; Costa et al., 2010). Genetic approaches aimed at stabilizing cristae shape have been successful in delaying or even blocking apoptosis induced by a plethora of intrinsic stimuli (Frezza et al., 2006; Yamaguchi et al., 2008; Costa et al., 2010). Nevertheless, cristae remodeling has been reckoned as a feedback mechanism in situ, occurring after caspase activation (Sun et al., 2007), and modelling studies have questioned the validity of cristae junctions as bottlenecks for cytochrome c diffusion (Gillick and Crompton, 2008; Scorrano, 2009; Tam et al., 2010). In addition, the precise mechanism by which proteins of the BCL-2 family can induce remodeling of the cristae is unclear: while outer membrane permeabilization seems to be dispensable, controversies remain as to the requirement for the BH3 domain (Scorrano et al., 2002; Yamaguchi et al., 2008; Landes et al., 2010). Our data contribute to clarify these issues, by identifying the requirement for a conserved stretch of aminoacids localized in the membrane anchor of the BH3-only proteins BID, Bnip3 and BimS that induce OPA1-dependent cristae remodeling (Scorrano et al., 2002; Yamaguchi et al., 2008; Landes et al., 2010). Mutations within this domain abrogated cristae changes and cytochrome *c* mobilization, as well as

the “metabolic” component of cell death in situ. On the other hand, as previously reported, the BH3 domain of BID is fully dispensable, in accordance with the lack of stringent conservation of this domain in Bnip3 that nevertheless is capable of triggering remodeling of the cristae. The α6 mutant of BID can also be regarded as a useful tool for in vivo studies aimed at dissecting the relative role of cristae remodeling in developmental and homeostatic apoptosis.

The notion that mitochondrial function is altered during apoptosis is well established. However, it remains unclear if dysfunction follows outer membrane permeabilization and is caused by the feedback of caspases on mitochondria, leading to the inactivation of important components of the NDUFS1 subunit of complex I (Ricci et al., 2004), or it is an intrinsic program that is elicited by the BH3 proteins relying the apoptotic signal to the organelle. While it is difficult to reconcile the former hypothesis with the maintenance of a morphological integrity of the outer membrane, the changes in organelle ultrastructure that accompany the release of protein cofactors can be associated with multiple dysfunctions. Remodeling of the cristae for example seems a natural candidate to impact on the function of the organelle, being these structures the preferential localization of respiratory chain complexes (Vogel et al., 2006). Our data indicate that when cristae are remodeled, respiratory efficiency is indeed reduced, ultimately lowering cellular proliferation when supported by mitochondria. A closer inspection revealed that the respiratory loss is caused by the disorganization of the quaternary structures of respiratory chain supercomplexes, supramolecular assemblies of the functional units of the respiratory chain (Schagger, 1995). The precise role of RCS in mitochondrial physiology is controversial, as it was until recently their existence, having RCS being considered for long artifacts of the BN PAGE technique used to isolate them (Schon and Dencher, 2009). Recently, direct respirometric evidence supports the ability of supercomplexes containing complex IV to transfer electrons from NADH to molecular O_2, identifying them as minimal respiratory units of the

mitochondrial membrane (Acin-Perez et al., 2008). RCS are indeed found in a variety of living organisms, from *E.coli* to yeast to plants (Lenaz and Genova, 2010); and assembly of RCS has been identified as a determining factor in the functional outcome of mtDNA complementation in models of mitochondrial diseases (D'Aurelio et al., 2006). However, several questions remained open: for example, what prompts RCS assembly? How relevant are RCS for respiration in vivo? Our data contribute to solve these conundrums: by using a genetic approach, we identify in cristae shape a required factor for RCS assembly. RCS are properly formed in situ only when cristae are not remodeled, be it for the action of the proapoptotic BID, or as a consequence of the genetic ablation of the master cristae shape regulator OPA1. By taking advantage of genetically defined models of mitochondrial changes during apoptosis, as well as of cells lacking *Opa1*, we could unravel a role for proper RCS assembly in mitochondrial respiratory efficiency in vitro and in vivo: when cristae are remodeled, cellular growth relying on mitochondria is impaired. These results offer a first answer to the question of whether RCS are important in mitochondrial physiology in vivo, and uncover a novel mechanism of mitochondrial dysfunction in apoptosis.

Our genetic dissection identified OPA1 as a key factor in the assembly of RCS. Previous reports identified this protein in complex with individual units of the respiratory chain and underlined the role of OPA1 in respiration funneled from complex I and in mitochondrial metabolism (Zanna et al., 2008), which is clearly deranged in patients suffering from dominant optic atrophy where Opa1 is mutated (Lodi et al., 2004). However, OPA1 did not seem to be an assembly factor for respiratory chain complexes and the defect in ATP production in cells from patients with *OPA1* haploinsufficiency has been left unexplained, since in these cells levels of mtDNA were stable (Zanna et al., 2008). On the other hand, impaired fusion in doubly *Mfn* deficient mice was reported to cause mitochondrial dysfunction by lowering mtDNA copy number (Chen et al., 2010). Careful dissection of the

role of mitochondrial fusion from that of cristae biogenesis in the definition of the respiratory ability of the organelle challenge this hypothesis. While RCS and efficiency of ATP production are impaired in *Opa1*$^{-/-}$ cells, *Mfn1*$^{-/-}$,*2*$^{-/-}$ cells that display the same impairment in mitochondrial fusion (Chen et al., 2005) and reduction in mtDNA copy number, but have normal cristae, are able to efficiently grow in galactose rich media, when ATP can be produced solely by mitochondria. Thus, the disorganization of RCS should be regarded as a potential mechanism of mitochondrial dysfunction that follows the morphological disorganization of the organelle. In this respect, it would be interesting to address if RCS are disassembled in multiple mitochondrial diseases where ultrastructural rearrangements occur.

EXPERIMENTAL PROCEDURES

Cell culture, transfection, virus production and transduction.

Opa1$^{-/-}$*, Opa1*$^{-/-}$*::*OPA1 *, Mfn1*$^{-/-}$*,2*$^{-/-}$ MEFs were cultured in DMEM containing 4.5mg/ml of glucose, supplemented with 10% of fetal bovine serum (FBS) and 50µg/ml of uridine as previously described (Song et al., 2007). DKO MEFs and HEK 293 cells were cultured in DMEM supplemented with 10% of FBS as described (Scorrano et al., 2003). When indicated, glucose in DMEM was substituted with 0.9mg/ml galactose. Cells were transfected using Transfectin (Biorad) following manufacturer's instruction.

Amphotrophic viruses were generated by co-transfecting the HEK 293 packaging cell lines with the packaging vector pIK and the required pMIG constructs as previously indicated (Cheng et al., 2001). Viral supernatant were retrieved and used to transduce DKO MEFs in the presence of 4µg/ml of Hexadimethrine Bromide (Sigma). Following an over-night transduction, the rate of infection obtained was typically around 60% as indicated by flow-cytometric detection of GFP expression.

Molecular biology

The retroviral vector pMIG-tBid was described previously (Cheng et al., 2001). Mutants BIDKKAA, G94E and G94EKKAA were generated by site-direct mutagenesis using KOD polymerase (Biolabs).

Assays of cell growth and death

The growth of wt, *Opa1*$^{-/-}$, *Opa1*$^{-/-}$::OPA1, *Mfns 1*$^{-/-}$*,2*$^{-/-}$ MEFs and of A22 fibroblasts was determined by plating $1.5x10^4$ cells in a 6 well-plate using the indicated medium. Viable cells as determined by Trypan Blue exclusion were counted daily for 5 days.

To determine the growth of DKO MEFs, following an over-night transduction with the indicated vectors, 1.5 x 10^4 cells were plated in a 6 well-plate and were grown in the indicated medium and the GFP positive cells were counted daily by flow cytometry.

For measurements of apoptosis, cells of the indicated genotype (15 x 10^4) grown in 6 well-plates were transfected with the indicated vector. After 48 hr apoptosis was measured by flow cytometric detection (FACSCalibur) as the percentage of Annexin-V-positive events in the GFP-positive population.

Recombinant protein expression

p7/p15 recombinant BID was produced, purified and cleaved with caspase8 as described in (Frezza et al., 2006) and was used at the final concentration of 3.2 pmolxmg^{-1} mitochondria.

In vitro mitochondrial assays

Mitochondria from liver of CD1 mice and from the indicated cell lines were isolated by standard differential centrifugation as described in (Frezza et al., 2007).

Cytochrome *c* redistribution and release in response to recombinant cBID was determinate as described in (Scorrano et al., 2002).

For respiration assays, mitochondria (1mg/mL) incubated for the indicated time in Experimental Buffer (EB: 150 mM KCl, 10 mM Tris Mops, 10 µM EGTA-Tris, 5 mM ATP) supplemented with 5 mM cytochrome *c* and 2mM of NADH in the presence of 3.2 pmol x mg^{-1} of cBID. Mitochondria were then transferred into the chamber of a Clark's type oxygen electrode and 5mM/2.5mM glutamate malate or 10mM succinate were added. Basal O_2 consumption was recorded (state 2) and after 2 minutes 100 µM ADP was added (state 3), followed by 2.5 µg/mL oligomycin to determine state 4 respiration.

Biochemistry

For protein crosslinking, mitochondria treated as indicated were treated with 1mM EDC or 1 mM BMH as previously described (Frezza et al., 2006). For SDS-PAGE, mitochondrial proteins (20 µg) were separated on a 3%-8% Tris-acetate or 4%-12% Tris-MES (NuPage, Invitrogen) polyacrilamide gel, transferred onto PVDF membranes (Biorad) and probed using the indicated primary antibodies and isotype matched secondary antibodies conjugated to horseradish peroxidase. Signal was detected with ECL (Amersham). Details on the antibodies used can be found in Supplemental data. Densitometry was performed by analyzing the optical density by using the Gel Pro Analyzer software.

Carbonate extraction was performed as previously indicated (Dimmer et al., 2008).

BN PAGE, 2D BN/BN PAGE, 2D BN/SDS PAGE.

To detect OPA1 oligomers, mitochondria were suspended in an appropriated volume of Buffer D (1M 6-aminohexanoic acid, 1.25% V/V digitonin, 50mM Bis-Tris-HCl, pH 7) at a final concentration of 10 mg/ml. Following centrifugation, the supernatant was collected and 5% Serva Blue G dye in 1M 6-aminohexanoic acid was added to one-third of the final volume of the sample. Equal amounts (100 µg) of mitochondrial proteins were separated in native condition on a 3%-14% gradient BNGE as described in (Schagger, 1995). To detect RCS, the concentration of digitonin in buffer D was 4% V/V and the gradient of the gel was 3-12%.

For 2D BN/BN PAGE, the lane from the first-dimension BN PAGE was cut from the gel and casted on top of a native 3-14% gradient gel in 1% (V/V) DDM. For 2D BN/SDS PAGE the lane from the first-dimension BN PAGE was cut from the gel, incubated for 1 hr at 25℃ in a loading buffer supplemented with 1% SDS a nd 1% β-mercaptoethanol, and then casted on top of an 8% or a 16.5% denaturing gel. After electrophoresis, the complexes

were electroblotted on a PVDF membrane and probed with the indicated antibodies (see Supplemental data for details).

To detect RCS from radiolabelled cells, samples were treated as described above and after electrophoresis, the gels were dried and the signal was detected following exposure for 3 up to 6 days.

Enzymatic in gel activity assay

Determinations of enzymatic in gel activity of complex I and complex IV were performed as described in (Zerbetto et al., 1997).

Pulse-chase experiments

Labeling of mtDNA-encoded proteins was performed with a [^{35}S] protein labeling mix. Cells were preincubated for 12 hrs in the presence of 40 mg/ml chloramphenicol and then exposed for 2 hrs to the [^{35}S] protein labeling mix (pulse) in the presence of 50µg/ml cycloheximide. Cells were washed for 4 times with PBS and cold DMEM and then cultured for the indicated time (chase, from 30 minutes to 48 hrs) prior to lysis and protein separation by BNGE.

Transmission electron microscopy

EM of cells and isolated mitochondria were performed exactly as in (Scorrano et al., 2002) and thin sections were imaged on a Tecnai-20 electron microscope (Philips-FEI). For morphometric analysis of mitochondrial cristae, cristae in each mitochondrion were counted and their number normalized for the area of the organelle using Metamorph as previously described (Costa et al., 2010).

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Figure Legends

Figure 1. Two conserved Lysines in α6-helix of BID are required for cristae remodeling

(A) Clustalw aligment between the aminoacid sequence of the BID hydrophobic α-6helix of BID (red) and Mastoparan an amphipathic peptide from wasp venom (green). Asterisk, identical residues; colon, high homology; dot, homology. Two conserved Lysine residues (K157, 158) are highlighted in grey

(B) Ribbon structure of BID showing the position of the two conserved K157,158 on the hydrophobic α-6helix.

(C) Carbonate extraction. Mouse liver mitochondria were treated with the indicated mutants of cBID for 30 minutes and then peripheral proteins were extracted by incubation in 0.1M Na_2CO_3 pH 11.3 for 30 minutes. Membrane and soluble fractions were recovered after centrifugation. Equal amount of proteins (10μg) were separated by SDS-PAGE and immunoblotted with the indicated antibodies. (T: total lysate, P: pellet, SN: surnatant).

(D) Mouse liver mitochondria were treated for the indicated times with the indicated mutants of cBID and cytochrome *c* release was measured. Data represent average ± SEM of 5 independent experiments.

(E) MLM treated with the indicated mutants of cBID for the indicated times were crosslinked with 1mM BMH for 30 min, spun and the pellet were separated by SDS-PAGE and immunoblotted using anti-BAK antibodies. Asterisks, BAK oligomers.

(F) Mitochondria were treated for 15 min with the indicated mutants of BID and then ascorbate over TMPD-driven respiration was determined as described. Data represent average ± SEM of 4 independent experiments.

(G) Mitochondria were treated as indicated (Ca^{2+}, 200 μM) and representative traces of NADH fluorescence changes following cytochrome b_5-dependent NADH oxidation were recorded.

(H) Representative EM fields of mitochondria treated for 15 minutes with the indicated BID mutants. Bar, 450 nm

Figure 2. Lysines 157,158 of BID are required for OPA1 oligomers disruption and efficient induction of apoptosis.

(A) MLM treated with the indicated mutants of cBID for the indicated times were crosslinked with 1mM EDC for 30 min, and equal amount (20 μg) of proteins in the pellet were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Asterisks indicate OPA1 oligomers.

(B) Kinetics of OPA1 oligomers destabilization by cBID. Experiments were as in (A). Data represent average ± SEM of 5 independent experiments.

(C-D) Blue native gel electrophoresis (BNGE) analysis of OPA1 oligomers in MLM treated as indicated for 30 min (C) or for the indicated times (D). Asterisks indicate high molecular weight complexes of OPA1.

(E) MEFs were transfected with pMIG containing the indicated insert. After 48 hours, cells were harvested and viability was determined. Data represents average ± SEM of 4 independent experiments.

Figure 3. Cristae remodeling affects the structure of respiratory chain supercomplexes

(A) RCR of MLM treated with cBID for the indicated times and then transferred into the chamber of a Clark's type oxygen electrode in EB supplemented where indicated with

5mM/2.5mM glutamate malate (GLU/MAL) or 10mM succinate (SUCC). Data represent mean ± SEM of 4 independent experiments. Inset, normalized RCR values.

(B) Experiments were as in (A) except that MLM were incubated with the indicated mutants of cBID for 15 minutes. The RCR of 4 independent experiments ± SEM are plotted.

(C) In gel activity of Complex I and Complex IV of mitochondria treated as indicated. The boxed areas are magnified 1.5X and autoequalized using the appropriate function of Photoshop to maximize visible differences in activity.

(D) Densitometric analysis of complex I and IV in gel activity assay. Experiments were as in C. Activity of the monomeric or supercomplex (I+III+IV) complex was measured by densitometry and normalized to untreated mitochondria. Data are mean ± SEM from 3 independent experiments.

(E) BN PAGE analysis of mitochondrial OXPHOS protein from DKO MEFs transduced as indicated, metabolically labelled for 2 hrs and lysed after 24hrs. Radioactivity was detected by exposing the dried and fixed gel for 1 week.

(F) Densitometric analysis of the ratio between monomeric and supercomplex complex I. Data represent mean ± SEM from three independent experiments performed as in (E).

(G) 2D BN/SDS PAGE analysis of mitochondrial OXPHOS proteins. Experiments were as in E except that following the first dimension the lanes were excised and proteins were further separated by a second dimension SDS-PAGE. The gels were dried and the signal was detected following 1 week of exposure.

Figure 4. Correct assembly of RCS requires OPA1.

(A) BN PAGE analysis of OXPHOS proteins in mitochondria isolated from MEFs of the indicated genotype. Equal amounts (100 µg) of proteins were separated in native conditions, transferred onto PVDF membranes and immunodecorated with the indicated

antibodies. Individual complexes and supercomplexes of the respiratory chain are indicated.

(B) 2D BN/SDS PAGE analysis of mitochondrial OXPHOS proteins. Experiments were as in A except that following the first dimension the lanes were excised and proteins were separated by a second dimension SDS-PAGE. The gels were dried and the signal was detected following 1 week of exposure. Individual complexes and supercomplexes of the respiratory chain as well as the single labeled proteins are indicated.

(C) Densitometric analysis of the ratio between the supercomplexes and the monomeric form of the indicated mtDNA encoded protein. Data represent average plotted ± SEM of 3 independent experiments.

(D) Respiratory Chain Supercomplexes (RCS) assembly assay. MEFs of the indicated genotype were metabolically labeled and chased for the indicated times. Equal amounts of proteins (100 µg) were separated by BN PAGE and radioactivity was detected in the fixed and dried gels for 1 week. Individual complexes and supercomplexes of the respiratory chain are indicated.

Figure 5. RCS are assebled in *Mfn1*$^{-/-}$,*2*$^{-/-}$ MEFs

(A) BN PAGE analysis of OXPHOS proteins. MEFs of the indicated genotype were metabolically labeled. Equal amounts of proteins (100 µg) were separated by BN PAGE and radioactivity was detected in the fixed and dried gels for 3 days. Individual complexes and supercomplexes of the respiratory chain are indicated.

(B) 2D BN/SDS PAGE analysis of mitochondrial OXPHOS proteins. Experiments were as in A except that following the first dimension the lanes were excised and proteins were separated by a second dimension SDS-PAGE. The gels were dried and the signal was detected following 1 week of exposure. Individual complexes and supercomplexes of the respiratory chain as well as the single labeled proteins are indicated.

(C) Respiratory Chain Supercomplexes (RCS) assembly assay. MEFs of the indicated genotype were metabolically labeled and chased for the indicated times. Equal amounts of proteins (100 µg) were separated by BN PAGE and radioactivity was detected in the fixed and dried gels for 1 week. Individual complexes and supercomplexes of the respiratory chain are indicated.

Figure 6. Disassembly of RCS impairs mitochondria-dependent cellular growth

(A-D) Cell growth curves of the indicated cell lines were cultured in DMEM supplemented with the indicated monosaccharides.

(E-F) Cell growth curves of DKO MEFs transduced with the indicated retroviruses and grown in DMEM supplemented with the indicated monosaccharides.

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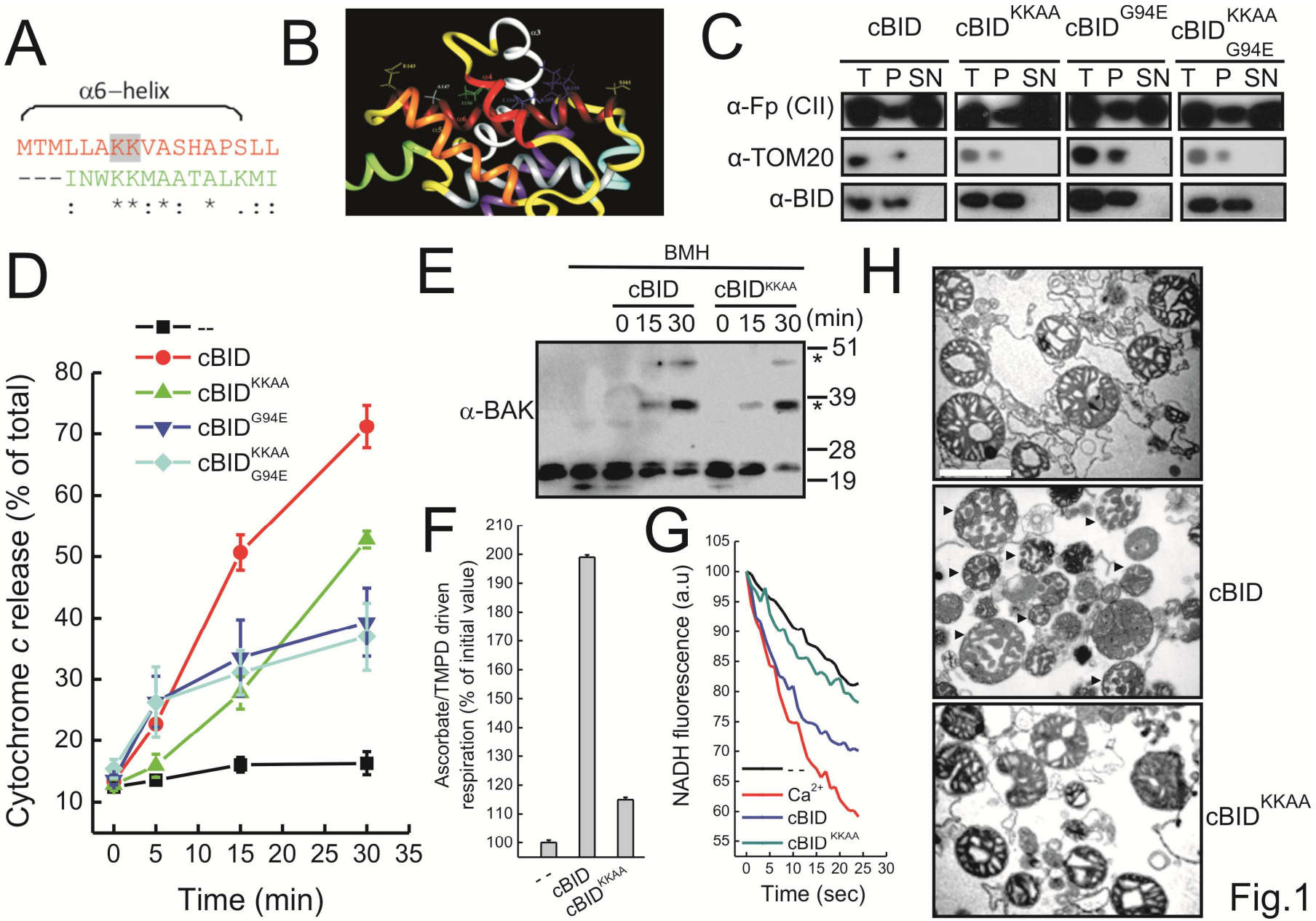
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cBID^KKAA G94E
T P SN
α-Fp (CII)
α-TOM20
α-BID
D
Cytochrome c release (% of total)
Time (min)
--
cBID
cBID^KKAA
cBID^G94E
cBID^KKAA G94E
E
BMH
cBID
cBID^KKAA
0 15 30 0 15 30 (min)
α-BAK
51
39
28
19
F
Ascorbate/TMPD driven respiration (% of initial value)
G
NADH fluorescence (a.u)
Time (sec)
Ca2+
cBID
cBID^KKAA
H
cBID
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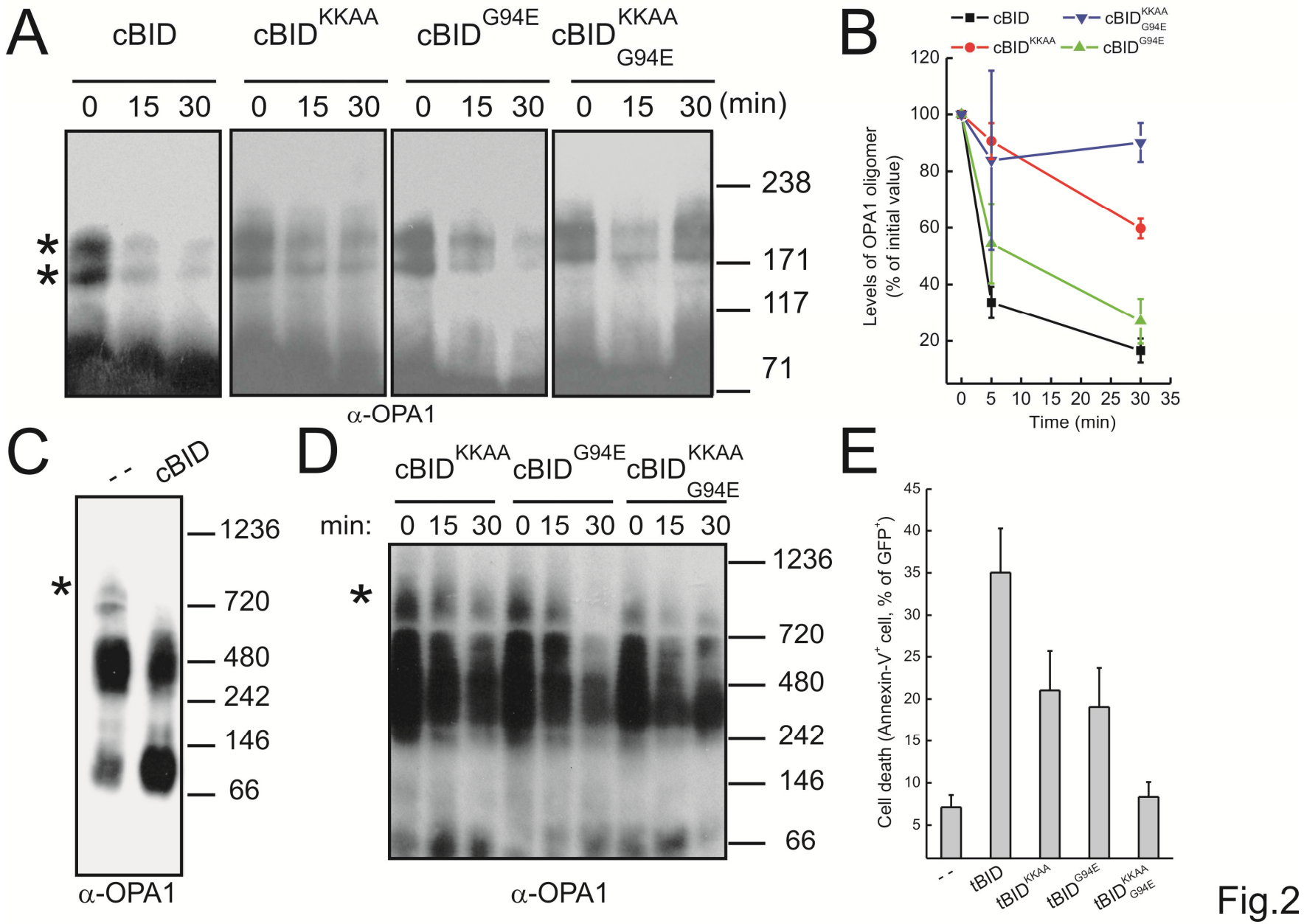
Fig.1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cBID^KKAA
cBID^G94E
cBID^KKAA G94E
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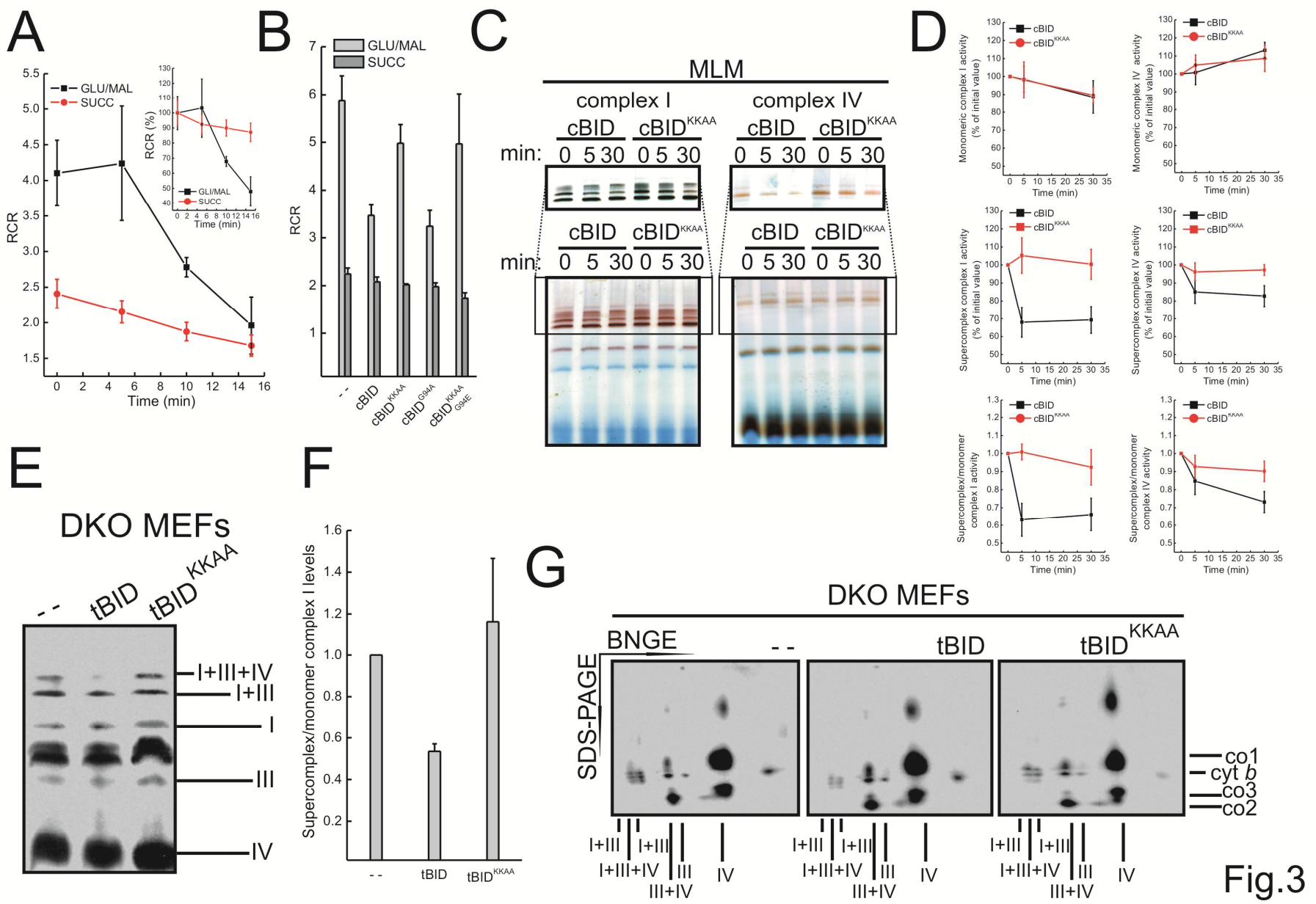
171
117
71
α-OPA1
B
cBID
cBID^KKAA
cBID^G94E
cBID^KKAA G94E
Levels of OPA1 oligomer (% of initial value)
Time (min)
C
-- cBID
1236
720
480
242
146
66
α-OPA1
D
cBID^KKAA
cBID^G94E
cBID^KKAA G94E
min: 0 15 30 0 15 30 0 15 30
1236
720
480
242
146
66
α-OPA1
E
Cell death (Annexin-V+ cell, % of GFP+)
-- tBID tBID^KKAA tBID^G94E tBID^KKAA G94E

Fig.2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GLU/MAL
SUCC
RCR
Time (min)
RCR (%)
B
GLU/MAL
SUCC
RCR
-
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cBID G94A
cBID KKAA G94E
C
MLM
complex I
complex IV
cBID
cBID KKAA
min: 0 5 30 0 5 30
D
Monomeric complex I activity (% of initial value)
Monomeric complex IV activity (% of initial value)
Supercomplex complex I activity (% of initial value)
Supercomplex complex IV activity (% of initial value)
Supercomplex/monomer complex I activity
Supercomplex/monomer complex IV activity
Time (min)
E
DKO MEFs
- -
tBID
tBID KKAA
I+III+IV
I+III
I
III
IV
F
Supercomplex/monomer complex I levels
- -
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G
DKO MEFs
BNGE
SDS-PAGE
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cyt b
co3
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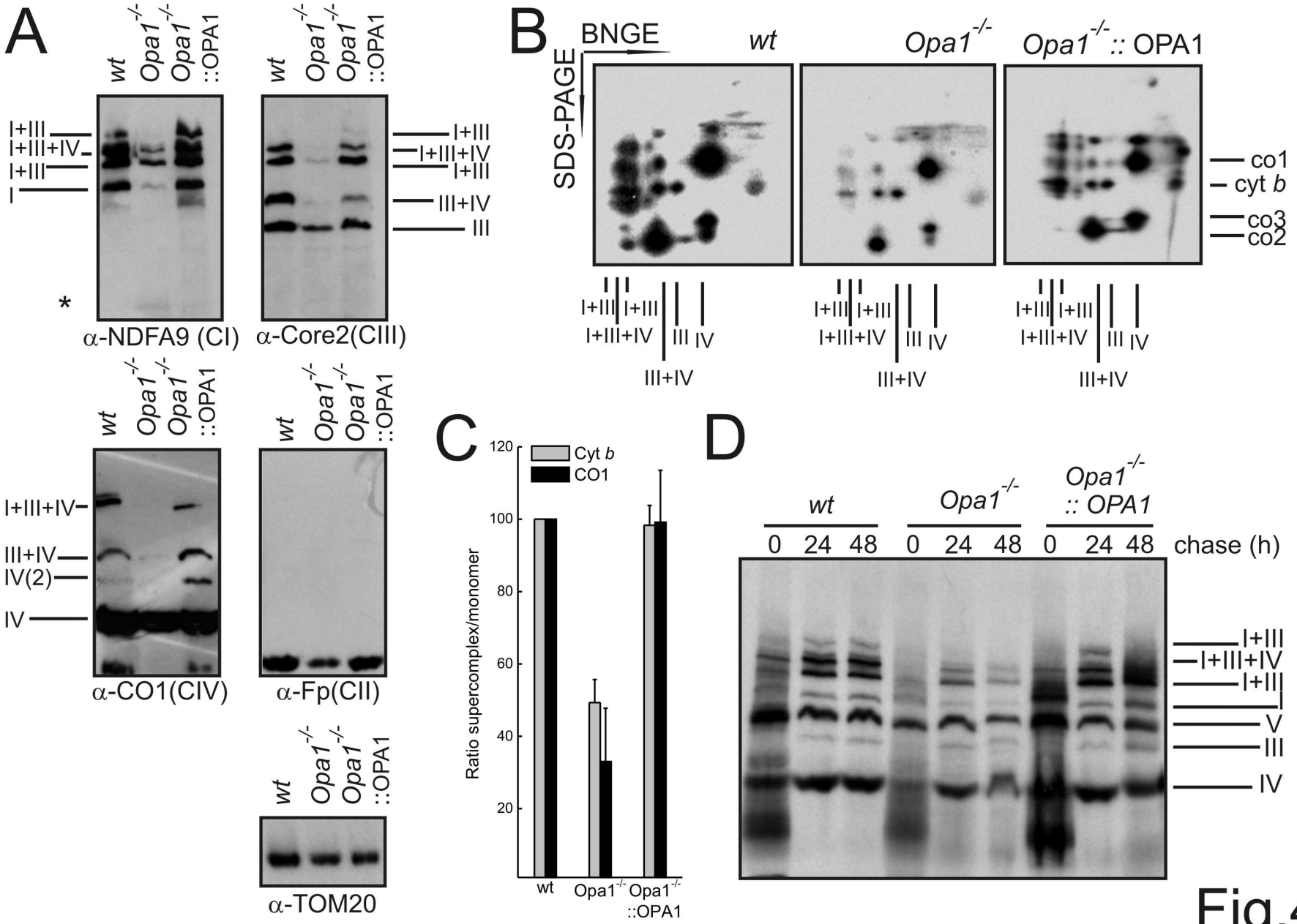
I+III+IV
III
III+IV
IV

Fig.3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wt
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I+III+IV
I+III
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*
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I+III
I+III+IV
I+III
III+IV
III
α-CO1(CIV)
α-Fp(CII)
IV(2)
IV
α-TOM20
B
BNGE
SDS-PAGE
wt
Opa1-/-
Opa1-/- :: OPA1
co1
cyt b
co3
co2
I+III
I+III+IV
III
IV
III+IV
C
Cyt b
CO1
Ratio supercomplex/monomer
120
100
80
60
40
20
wt
Opa1-/-
Opa1-/- ::OPA1
D
wt
Opa1-/-
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chase (h)
I+III
I+III+IV
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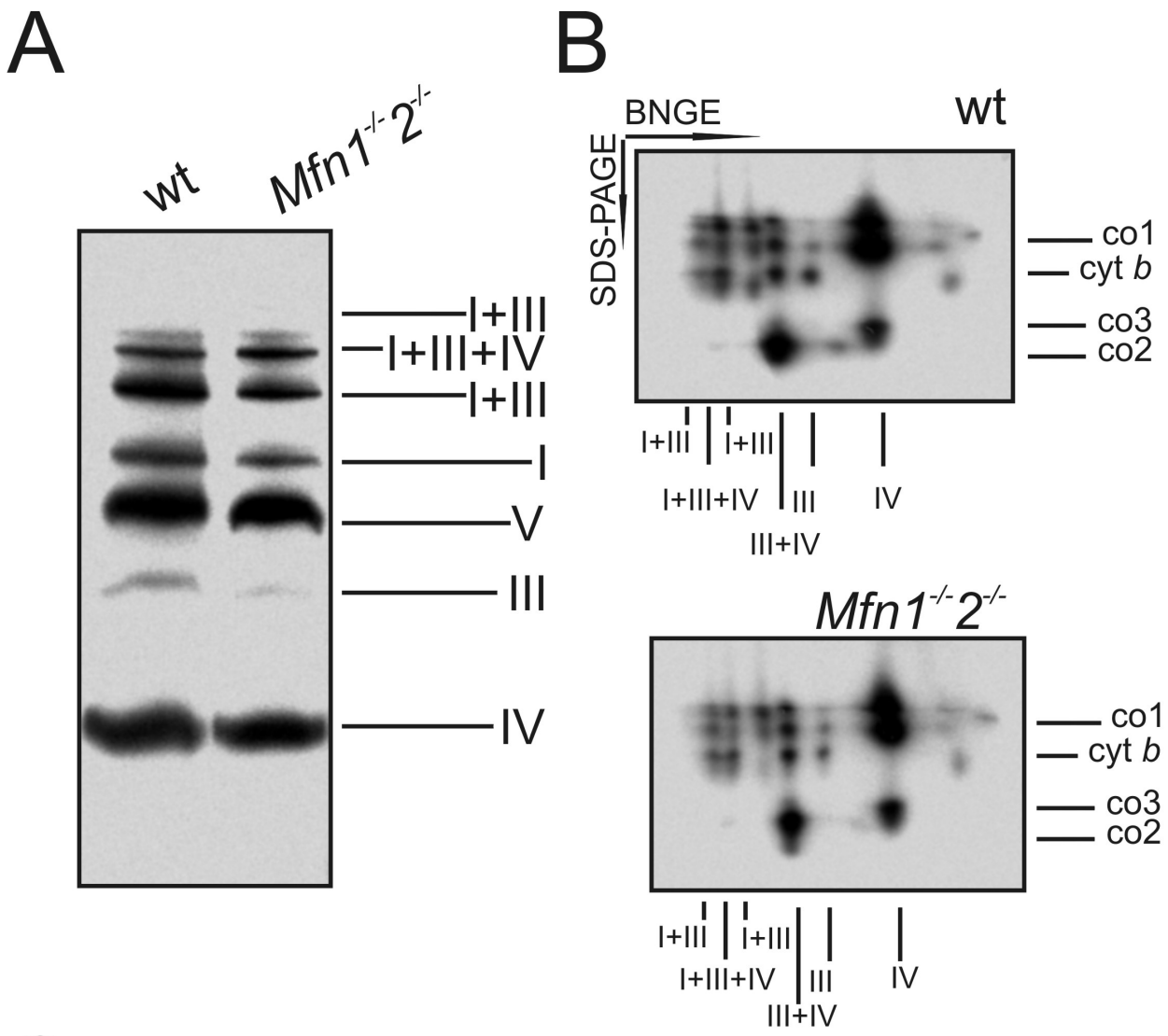
V
III
IV

Fig.4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B
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wt
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cyt b
co3
co2
I+III
I+III
I+III+IV
III
IV
III+IV
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co1
cyt b
co3
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III
IV
III+IV
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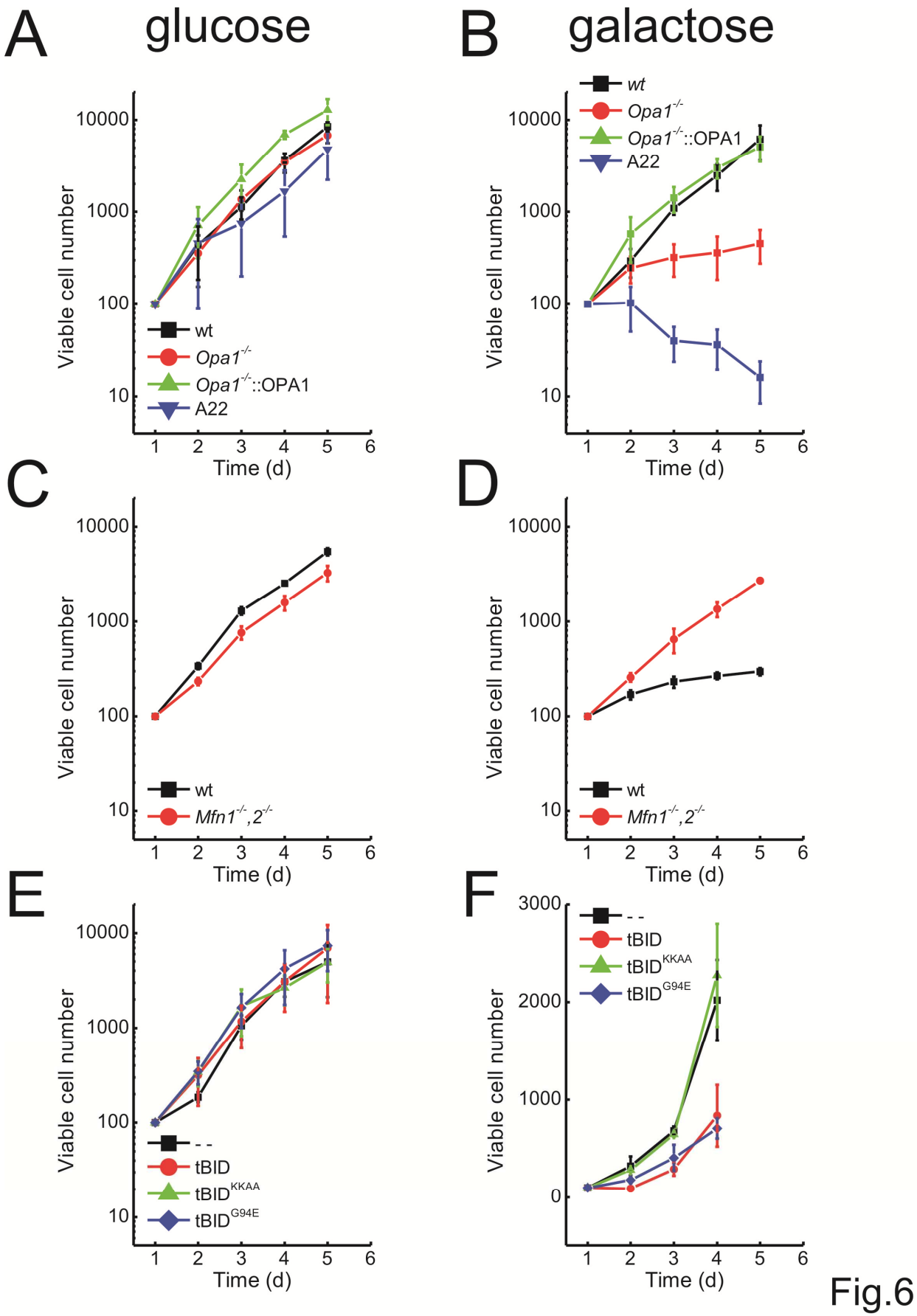
I
V
IV

Fig.5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glucose
B
galactose
C
D
E
F
Viable cell number
Time (d)
wt
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Opa1-/-::OPA1
A22
Mfn1-/-,2-/-
- -
tBID
tBIDKKAA
tBIDG94E

Fig.6

Opa1-dependent cristae remodeling disassembles respiratory chain supercomplexes, triggering apoptotic mitochondrial dysfunction

Sara Cogliati, Christian Frezza, Patricio Fernandez-Silva, Ester Perales-Clemente, Ligia C. Gomes, Jose A. Enriquez and Luca Scorrano

Supplementary online material

Supplementary experimental procedures

Molecular biology

The mutants of BID were generated by site-direct mutagenesis using the following primers: to generate BID^{KKAA} forward 5′-ACA ATG CTG TTG GCC GCC GCC GTG GCC AGT CAC-3′ and reverse 5′-GTG ACT GGC CAC GGC GGC GGC CAA CAG CAT TGT-3′.

to generate BID^{G94E} forward 5′-CTC GCC CAA ATA GAA GAT GAG ATG GAC CAC AAC-3′ and reverse 5′-GTT GTG GTC CAT CTC ATC TTC TAT TTG GGC GAG-3′.

Enzymatic in gel activity assay

The complex IV activity in gel was detected by incubating the BN PAGE gel at room temperature in 50mM Buffer Phosphate pH 7.4 supplemented with 2,5 mg/ml DAB (3,3'- diaminobenzidine tetrahydrochloride) (Sigma), 5mg/ml cytochrome *c* heart bovine (Sigma), 0.38mg/ml saccarose and 20 µg/ml catalase (Sigma). The reaction was stopped with 10% (V/V) acetic acid. The complex I activity was detected by incubating 0.1 mg/ml NADH (Sigma), 2.5 mg/ml NTB (NitroBlueTetrazoline) (Sigma) dissolved in 2mM Tris-HCl pH 7.4 the reaction was stopped with 10% (V/V) acetic acid.

Complex IV dependent- O_2 consumption

MLM (1mg/mL) were incubated for the indicated time in EB added of 5 mM cytochrome *c* in the presence or in absence cBID for 15 min. Mitochondria were treated with 1nmol/mg Antimycin A and 2nM rotenone and then they were transferred into the chamber of a Clark's type oxygen electrode. After 2min

10mM Ascorbate and 300mM TMPD were added and the Complex IV-dependent O_2 consumption rate was measured.

Release of soluble OPA1

Mitochondria isolated from wt and DKO MEFs were incubated with cBID for the indicated times, centrifuged for 10 min at 12000g at 4℃ and proteins retrieved in the pellet (t) and supernatant (s) were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

Measurement of mitochondrial DNA copy number

Total cellular DNA was isolated using phenol/chlorophorm 24:25 (v/v) and was amplified using specific oligodeoxynucleotides for *mt-Co2* and *Sdha* by real-time PCR using Platinum SYBR Green qPCR Supermix (Invitrogen) following manufacturer's indications. The mtDNA copy number *per cell* was calculated using *Sdha* amplification as a reference for nuclear DNA content.

Antibodies

For the immunoblots, the following primary antibodies were employed: Monoclonal anti-OPA1 (1:1000 BD Pharmingen), rabbit polyclonal anti-BAK-NT (1:1000 Upstate), monoclonal anti-Fp subunit (1:5000), anti-NDUF9 subunit (1:1000), anti-Core2 subunit (1:1000), anti-CO1subunit (1:1000) (MitoScience), rabbit polyclonal anti-TOM20 (1:5000 Santa Cruz), rabbit polyclonal anti-BID (1:1000, a kind gift of A. Gross, Weizmann Institute, Rehovot).

Legends to supplementary figures

Figure S1. Conservation of a Mastoparan-like domain in BID, Bnip3 and BimS.

(A) ClustalW alignment of the aminoacidic sequence of the transmembrane domains of BimS and Bnip3 and the α6-helix of BID. **(B)** ClustalW aligment of the aminoacidic sequence of BID of the indicated species. Aminoacid similarity and identity are color coded.

Figure S2. cBID destabilizes HMW oligomers of OPA1.

(A) 2D BN/SDS PAGE analysis of the OPA1 oligomers and complex IV. MLM were treated as indicated for 30 minutes. Equal amount (50µg) of proteins were separated in native condition and then the lanes were excised and proteins separated by a second dimension SDS-PAGE. After immunoblotting, the proteins were immunodecorated with the indicated antibodies. Asterisk indicates high molecular weight complexes of OPA1.

(B) 2D BN/BN PAGE analysis of OPA1 oligomers in MLM treated as indicated for 30 minutes. The first dimension BN PAGE was performed as in (A). Then the lane was cut and proteins were separated by a second dimension native page. The complexes were immunoblotted and probed with the indicated antibodies.

Figure S3. BAX and BAK are required for OPA1 release but not for disruption of OPA1 oligomers during apoptosis.

(A) Isolated mitochondria from the indicated cell lines were treated with cBID for the indicated times. Then they were crosslinked with 1 mM EDC and equal

amount (20 µg) of proteins in the pellet were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Asterisks indicates the high molecular weight complexes of OPA1.

(B) Release of soluble form of OPA1. Mitochondria isolated from the indicated cell lines were treated as described. Following centrifugation, the proteins retrieved in the pellet (p) and surnatant (sn) were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

Figure S4. cBID does not impair complex IV-dependent respiration.

MLM (1mg/mL) incubated in the presence or in absence of cBID for 15 minutes were treated with 1nmol/mg antimycin A and 2nM rotenone. Then they were transferred into the chamber of a Clark's type oxygen electrode in EB supplemented with 10mM Ascorbate and 300mM TMPD.

Figure S5 : Assembly of RC complexes is not affected in *Opa1*$^{-/-}$ cells.

(A) Densitometric analysis of radioactivity incorporated in complex IV and V following chase. Data represent avarage ± SEM of 3 independent experiments.

(B) Densitomeric analysis of the ratio between the radioactivity incorporated in RCS and complex V. Data represent avarage ± SEM of 3 independent experiments.

Figure S6. Respiratory Chain Supercomplexes assembly assay.

Respiratory Chain Supercomplexes (RCS) assembly assay. The experiment was performed as described before in Fig. 4D except that solubilization of mitochondria was performed using 1% (v/v) DDM.

Figure S7. Mitochondrial DNA copy number is reduced in *Opa1*$^{-/-}$ and *Mfn1*$^{-/-}$,*2*$^{-/-}$ MEFs.

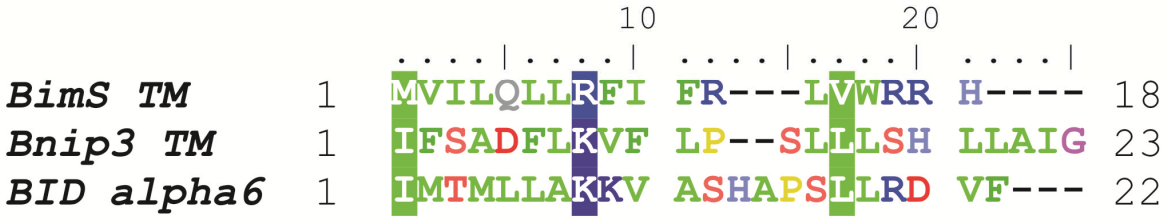
mtDNA was amplified by RT-PCR from total DNA of *Opa1*$^{-/-}$ (A) and *Mfn1*$^{-/-}$,*2*$^{-/-}$ (B) cells as described. Data are normalized to the mtDNA copy number of the respective wt MEFs and represent mean ± SEM of 5 dependent experiments.

Figure S8. Cristae biogenesis is impaired in*Opa1*$^{-/-}$ but not in *Mfn1*$^{-/-}$,*2*$^{-/-}$ MEFs

(A) representative EM images of mitochondria from cells of the indicated genotype.

(B) morphometric analysis of cristae biogenesis. Experiments were as in (A). TEM images of randomly selected fields from cells of the indicated genotype were acquired and the number of cristae in each counted mitochondrion was normalized for the area of the organelle. Data are mean ± SEM of 3 independent experiments. Bar, 1µM

A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20
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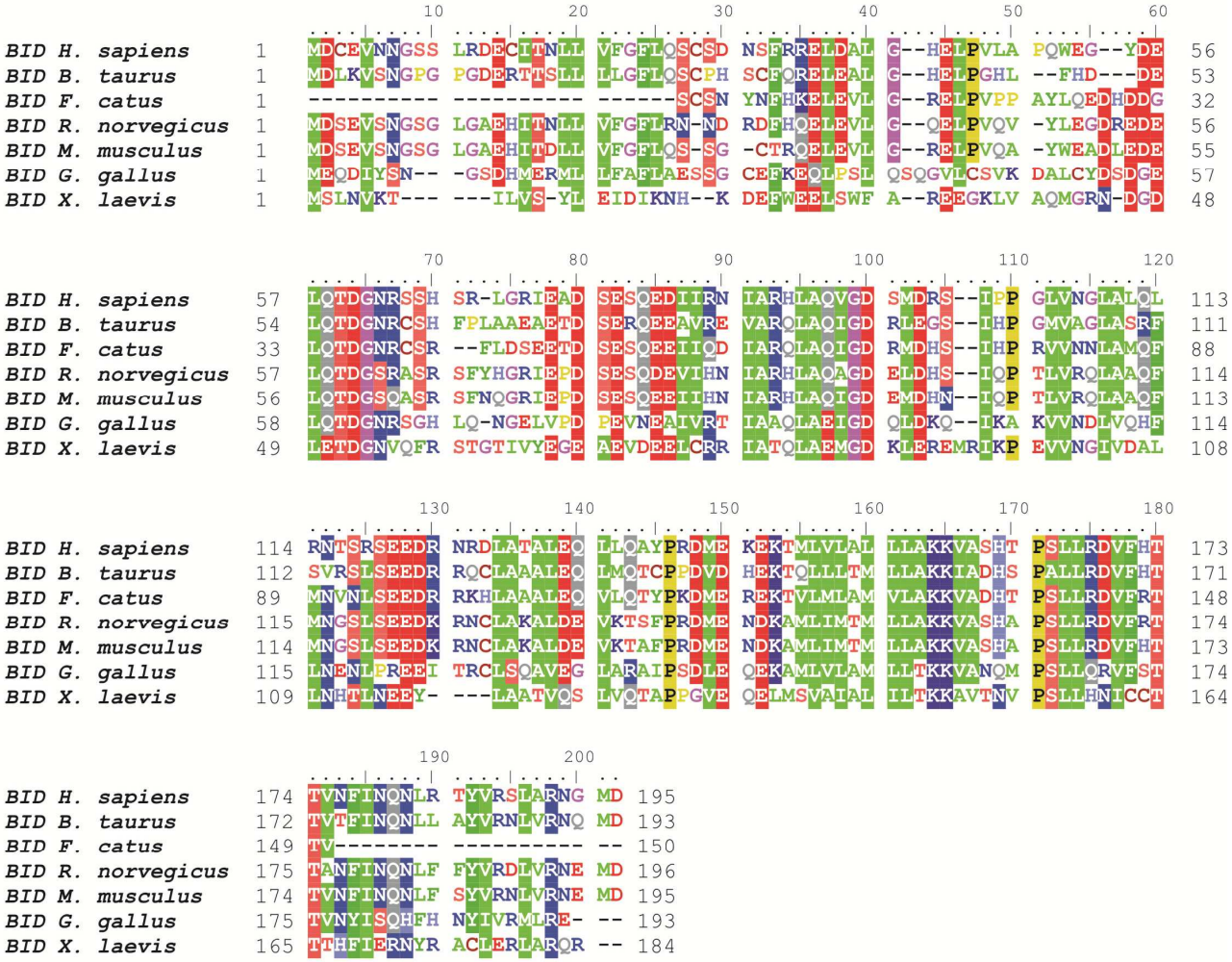
B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BID R. norvegicus 1 MDSEVSNGSG LGAEHITNLL VFGFLRN-ND RDFHQELEVL G--QELPVQV -YLEGDREDE 56
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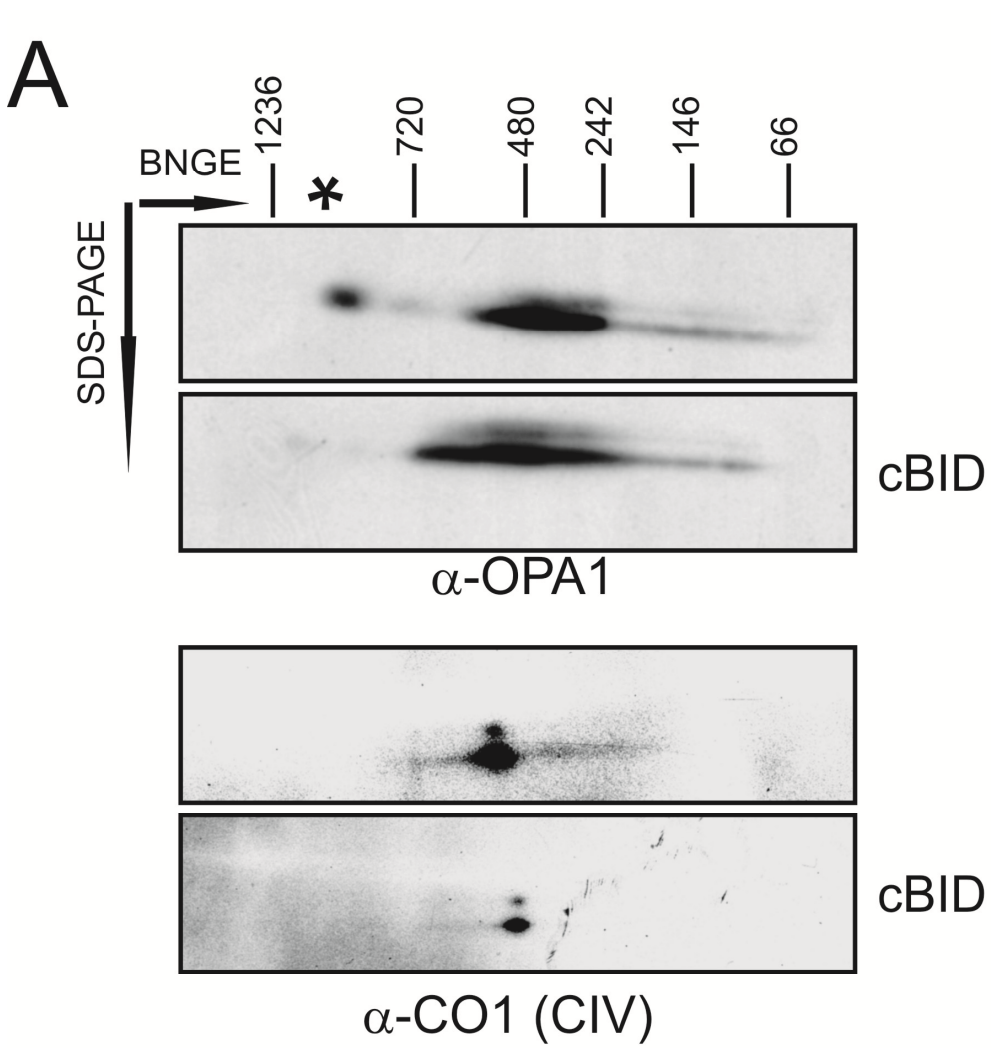
S1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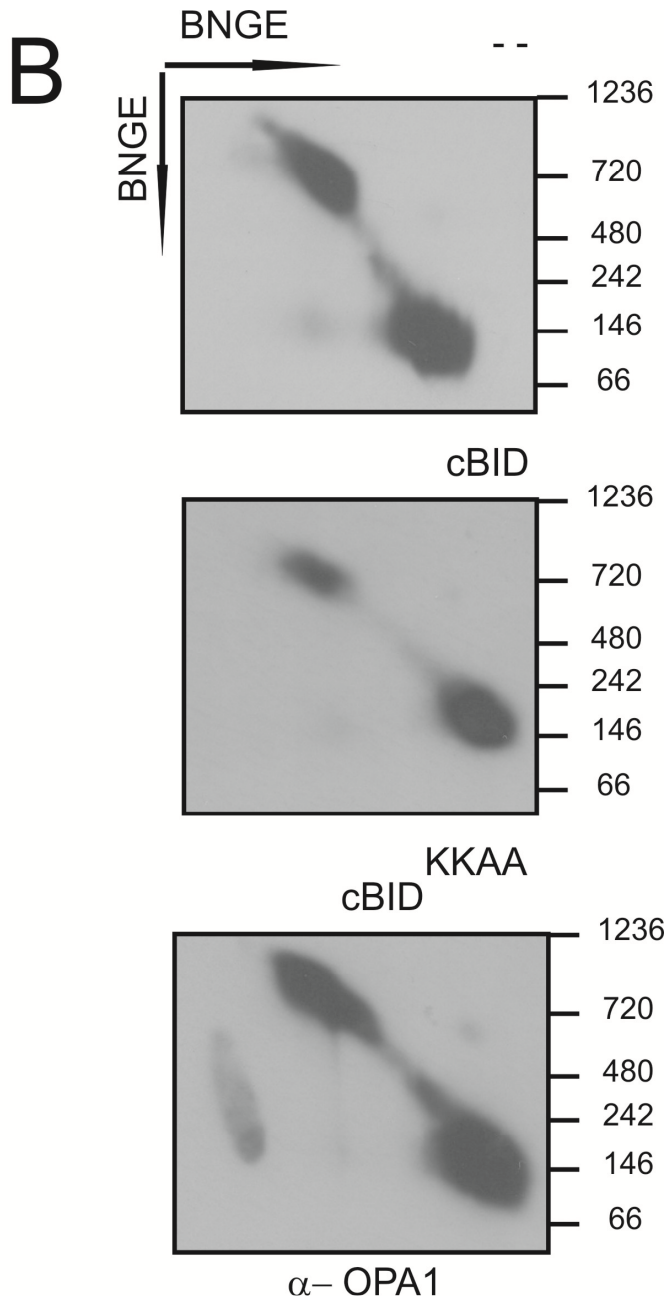
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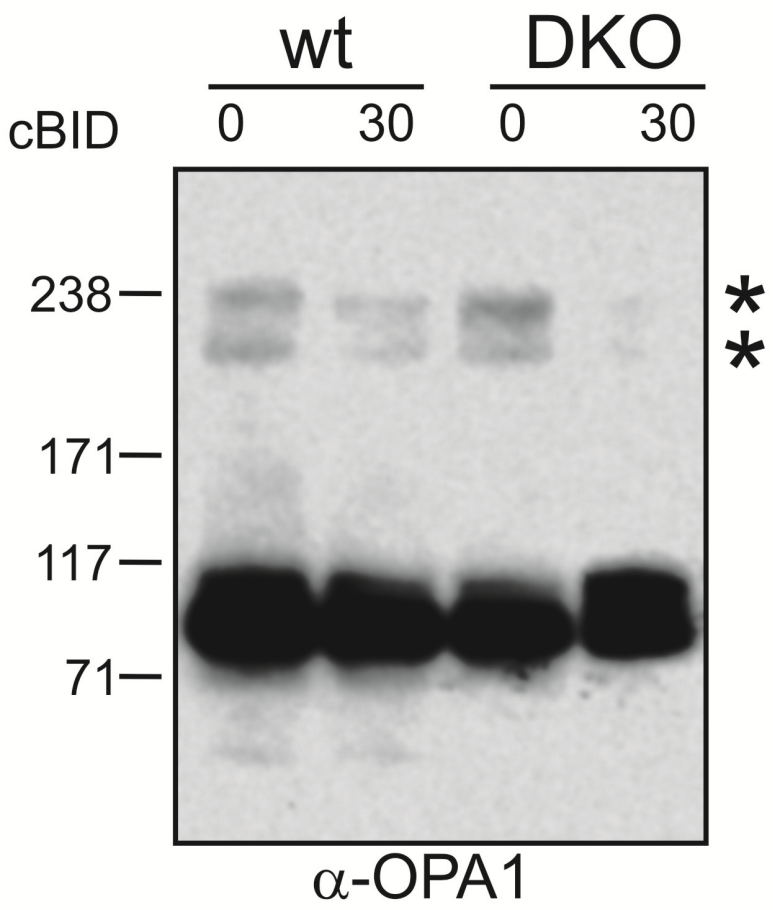
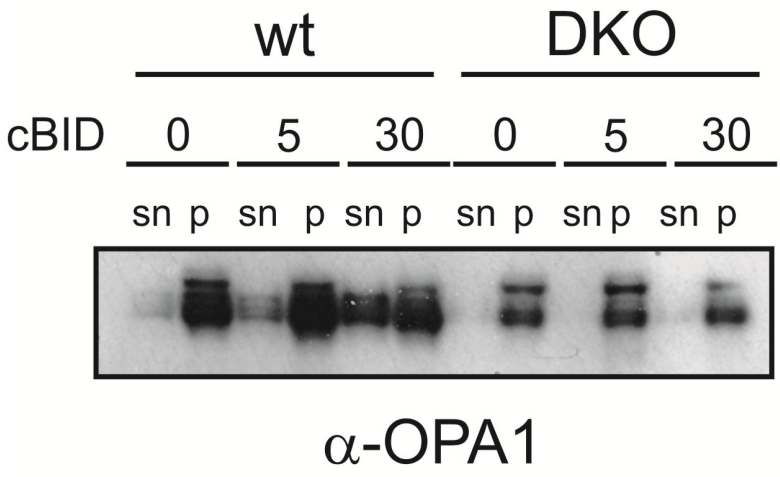
α– OPA1

S2

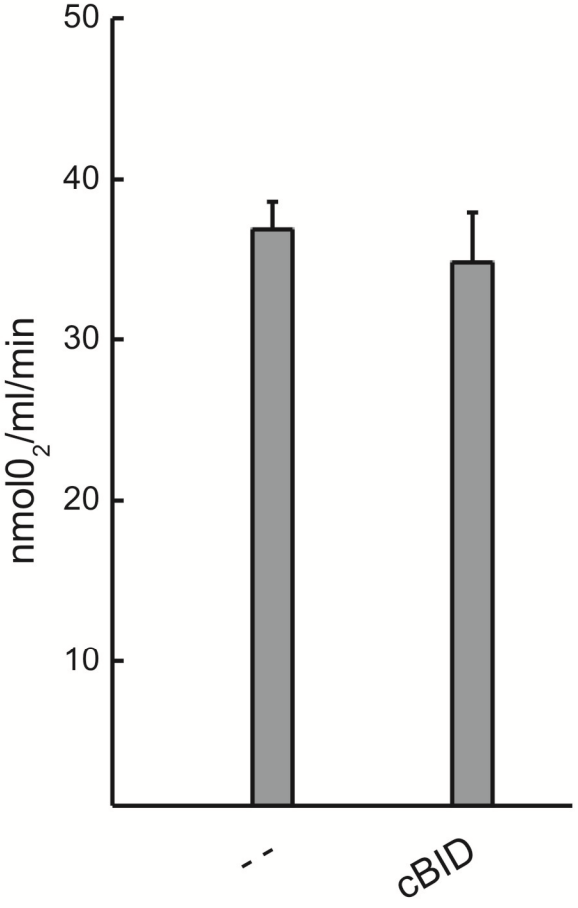
A


wt
DKO
cBID
0
30
0
30
238
171
117
71
*
*
α-OPA1

B

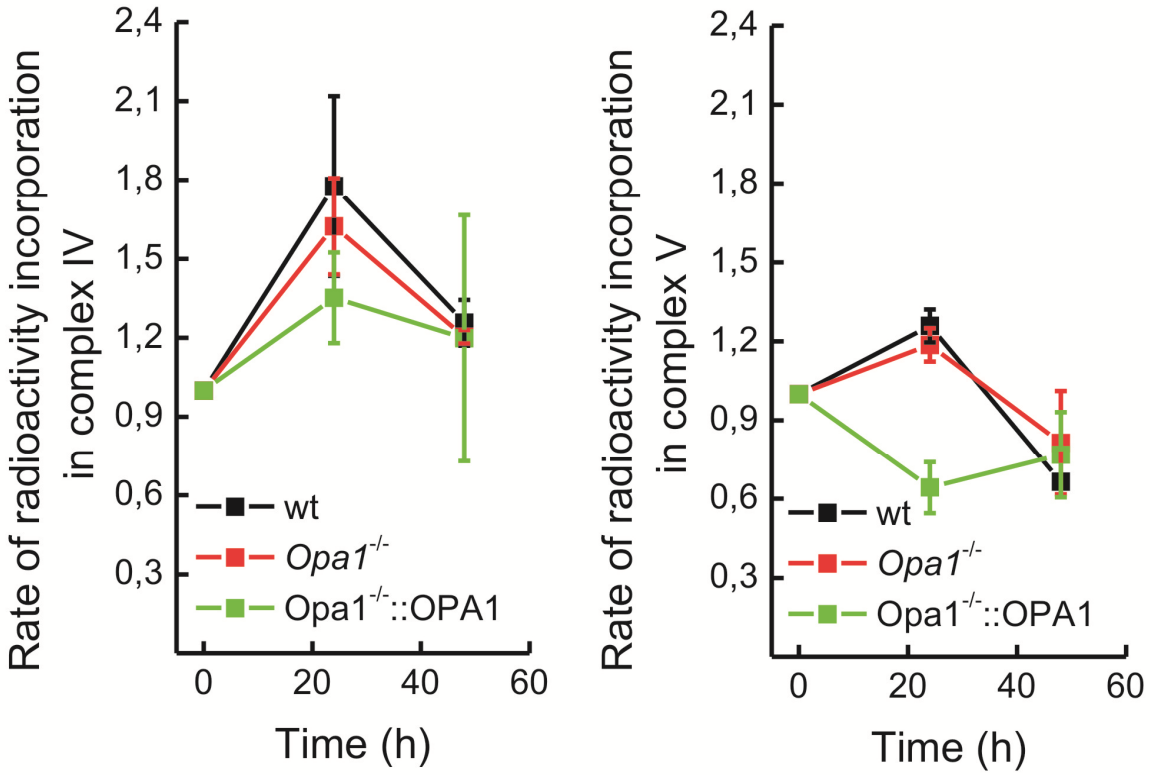

wt
DKO
cBID
0
5
30
0
5
30
sn p sn p sn p sn p sn p sn p
α-OPA1

S3

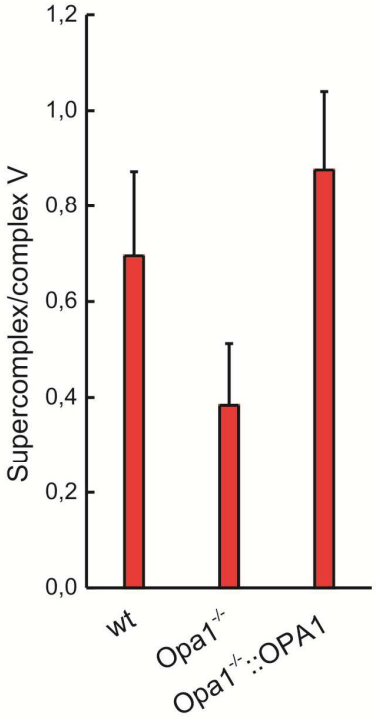


S4

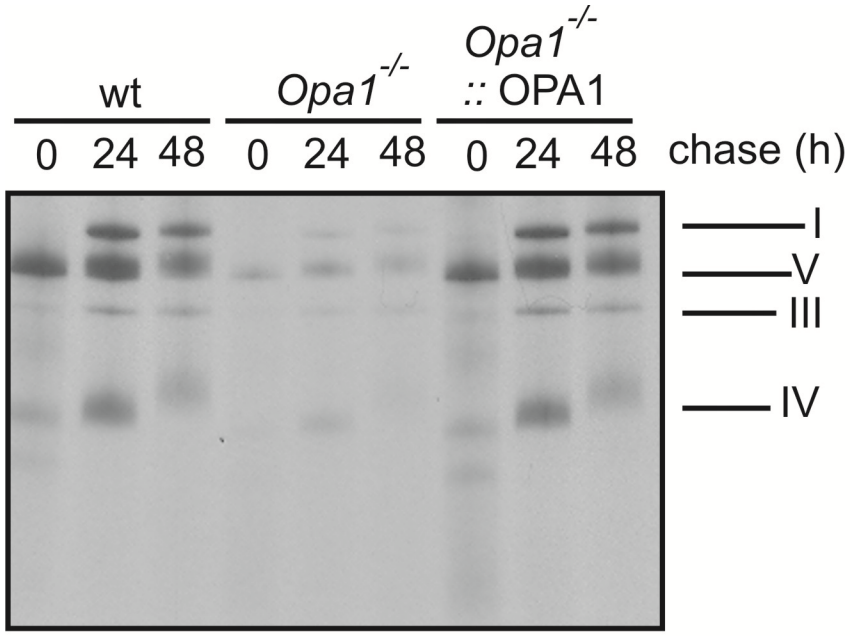
A


Rate of radioactivity incorporation in complex IV
2,4
2,1
1,8
1,5
1,2
0,9
0,6
0,3
wt
Opa1-/-
Opa1-/-::OPA1
0
20
40
60
Time (h)
Rate of radioactivity incorporation in complex V
2,4
2,1
1,8
1,5
1,2
0,9
0,6
0,3
wt
Opa1-/-
Opa1-/-::OPA1
0
20
40
60
Time (h)

B

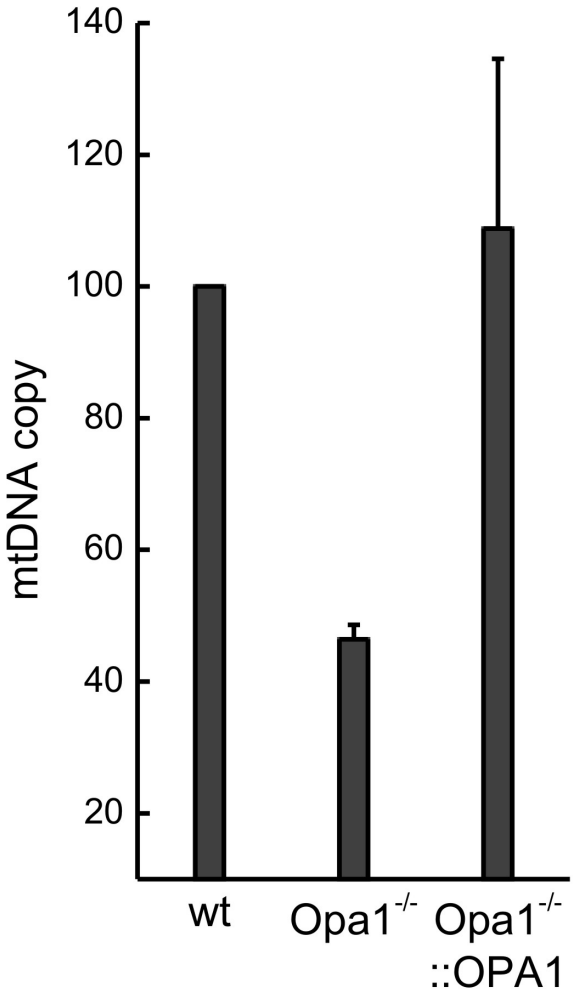

Supercomplex/complex V
1,2
1,0
0,8
0,6
0,4
0,2
0,0
wt
Opa1-/-
Opa1-/-::OPA1

S5

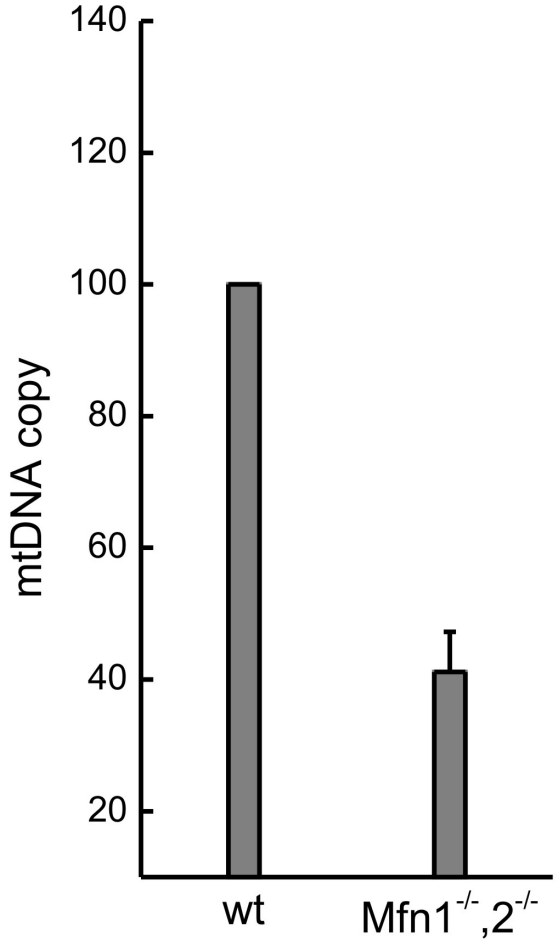


S6

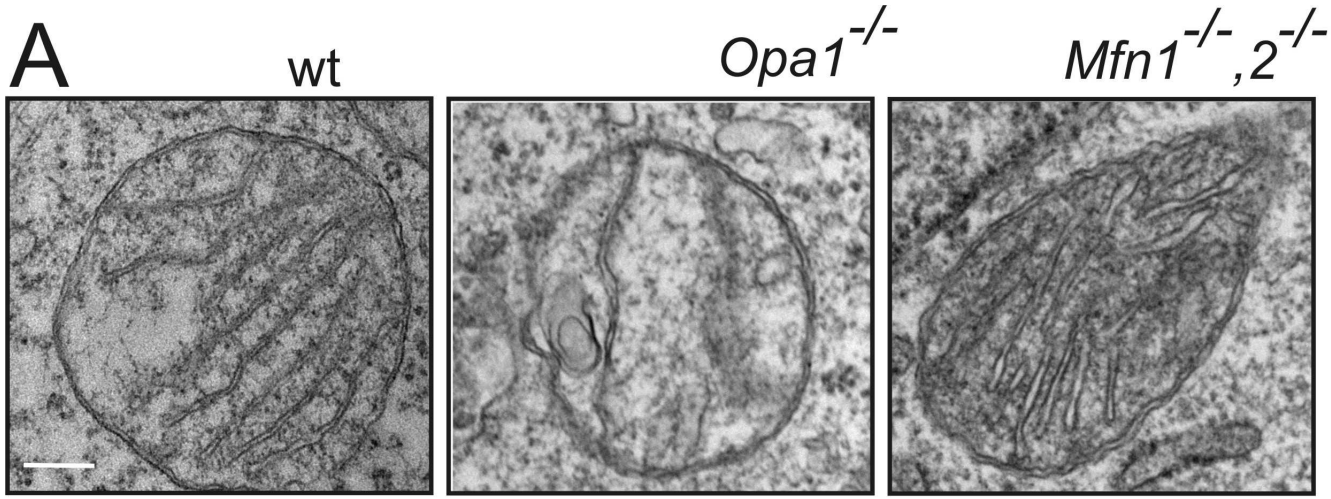
A

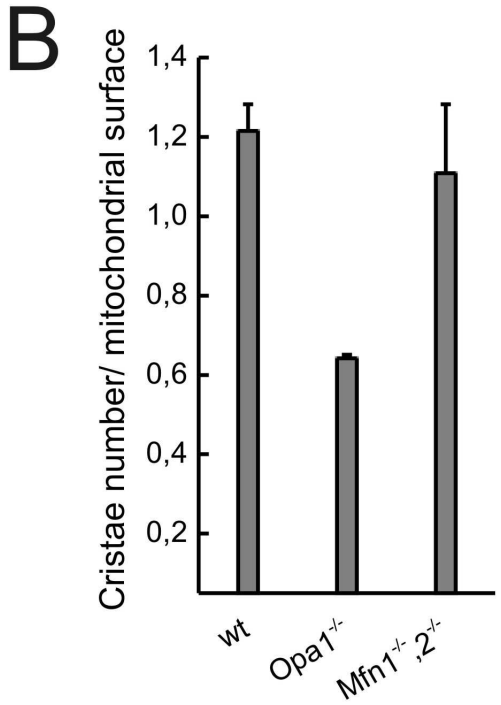

140
120
100
80
60
40
20
mtDNA copy
wt
Opa1-/-
Opa1-/-
::OPA1

B


140
120
100
80
60
40
20
mtDNA copy
wt
Mfn1-/-,2-/-

S7


A
wt
Opa1-/-
Mfn1-/-,2-/-


B
Cristae number/ mitochondrial surface
1,4
1,2
1,0
0,8
0,6
0,4
0,2
wt
Opa1-/-
Mfn1-/-,2-/-

5. Conclusions

In this thesis we addressed the role of cristae remodeling and of OPA1-dependent cristae biogenesis on mitochondrial metabolism. Our data indicate that (i) the shape of cristae is important for the assembly of respiratory chain supercomplexes (RCS) and that (ii) both apoptosis and genetic ablation of *Opa1* impacts on the assembly and on the activity of respiratory chain supercomplexes; ultimately determininig the efficiency of the cell when it is forcedto the utilize mitochondria for energy conversion. The cristae remodeling induced by proapoptotic BH3 only BCL2 family proteins is an important step in cytochrome *c* mobilization, but its roles on mitochondrial physiology remains unknown as well as the mechanism by which these BCL2-proteins induce cristae remodeling. To address these crucial questions we have generated a mutant of BID in the α6-helix and we demonstrated that this region is responsible of the cristae remodeling. Exploiting this molecular tool we demonstrated that cristae remodeling affects mitochondrial respiration by impacting the structure of RCS. Moreover, the molecular mechanisms that control RCS structure and assembly are unclear. Reasoning on the fact that OPA1, whose oligomers are affected during cristae remodeling, has a role in controlling cristae shape, we tested whether the RCS structure could be disturbed by the absence of OPA1. Indeed in *Opa1*$^{-/-}$ cells the individual complexes of respiratory chain fail to be assembled into RCS, resulting in an aberrant RCS composition. The physiological relevance of RCS disassembly was highlighted by the severe impairment in cell growth when it depends on mitochondrial substrates. In conclusion, our data represent a step forward in the comprehension of the effects of cristae remodeling during apoptosis and of the molecular mechanisms controlling the assembly of RCS. They indicate that the shape of the cristae which is controlled by the dynamin like protein OPA1 is an essential element for the correct assembly of RCS and as a consequence OPA1 results to be a key modulator of the RCS structure and mitochondrial respiratory efficiency.

Another consequence of our results is that our genetic systems allows to dissect the functional role of RCS in situ and hightlight their importance for cell growth. To our knowledge, ours is the first genetic evidence that proper assembly of the RCS is requried for efficient cell growth. This could open new perspective to explain tissue selectivity of some mitochondrial diseases: if the common underlying defect is a disassembly of RCS it might be tempting to speculate (and worth to investigate) that the most affected tissues are the ones where RCS are key to support the mostly mitochondrial ATP production. In this respect, forcing cristae biogenesis could be an

interesting approach to ameliorate respiratory efficiency and hence natural history of these diseases.

Finally, we should point out that the role of mitochondrial dysfunction in apoptosis ahas been widely debated. Two opposing models postulate that mitochondrial functional changes are either an intrinsic component of the pathway of cytochrome c release, or a byproduct of caspase activation following the release of cytochrome c. Without entering into the details of this dispute, we would like to point out that the combination of genetics and metabolic studies presented in this thesis underline the importance of topology of the inner membrane to provide the proper platform for efficient oxidative phosphorylation. In fact, when we induce cristae remodelling in Bax, Bak deficient cells that can't release cytochrome c during apoptosis and that are forced to produce ATP via mitochondria, we observed a remarkable reduction in cell growth, suggestive of reduced ATP production and hence of mitochondrial dysfunction.

In conclusion, the combination of genetics and mitochondrial physiology has been instrumental to address the relationship between cristae shape, RCS assembly, mitochondrial function and apoptosis.

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