C11orf83, a mitochondrial cardiolipin-binding protein involved in bc1 complex assembly and supercomplex stabilization

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Abstract


Reference


DOI : 10.13097/archive-ouverte/unige:108015
URN : urn:nbn:ch:unige-1080158
C11orf83, a mitochondrial cardiolipin-binding protein involved in bc1 complex assembly and supercomplex stabilization.

THESE
présentée à la Faculté des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences, mention biologie

par
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Genève
2015
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Remerciements

Je tiens tout d’abord à remercier les membres du jury, le Dr. Elena Ziviani, le Pr. Amos Bairoch, le Pr. Jean-Claude Martinou et le Dr. Riekelt Houtkooper, qui m’ont fait l’honneur de lire et d’évaluer ce travail.

Merci à mon directeur de thèse, le Pr. Amos Bairoch, et à mon superviseur de thèse le Dr. Lydie Lane pour m’avoir accueillie au sein du groupe CALIPHO et donné l’opportunité de faire cette thèse. Merci pour le temps que vous m’avez consacré, vos précieux conseils et pour toutes nos discussions enrichissantes. Merci également pour la confiance que vous m’avez accordée tout au long de ces années. Ce fut un plaisir d’être une CALIPHETTE !

Merci aussi à mon co-directeur de thèse, le Pr. Jean-Claude Martinou pour sa disponibilité et les précieuses suggestions apportées lors de nos rencontres. Merci également de m’avoir acceptée au sein de votre laboratoire afin de découvrir les TLC avec le Dr Etienne Raemy que je remercie aussi pour m’avoir appris cette technique et pour avoir pris de son temps pour réaliser les TLC de cette étude.

Je n’oublie pas le Pr. Dominique Soldati-Favre et le Pr. Denis Martinvalet qui ont eu la gentillesse d’accepter d’être mes « Parrains ». Merci au Pr. Denis Martinvalet pour sa disponibilité, ses précieux conseils et son intérêt vis-à-vis de mon travail.

Je tiens à remercier le Dr. Frédéric Vaz et le Pr. Michelangelo Foti d’avoir accepté de collaborer avec nous sur ce projet et d’avoir ainsi participé à la publication de nos données.

Je tiens à remercier très sincèrement Rachel Porcelli avec qui j’ai eu la chance de travailler pendant plus de 3 ans sur ce projet. Merci d’avoir accepté de travailler avec moi aussi bien au labo qu’en chambre froide! Tu as su rendre ces nombreuses heures dans le froid très drôles. Merci pour ton soutien sans faille, et ton optimisme qui m’ont plus d’une fois permis de reprendre les pipettes. Ton rire, ta bonne humeur et bien sûr nos discussions vont me manquer.

Je souhaite remercier également Irène, Lisa, Camille, Fabiana, Aurore, Paula, Franck et David qui sont, ou ont été, un jour CALIPHO. Vous avez tous contribué à la bonne ambiance au labo, merci pour nos fous rires entre deux manips. Merci tout spécialement à Irène pour sa gentillesse, sa disponibilité et son écoute.

Merci également aux membres du CALIPHO groupe, côté bio-informatique, pour leur gentillesse et leur bonne humeur communicative.

Merci aux « Hartley », Marianne, Séverine, Fabrice, Hubert et Alexandre pour leur gentillesse, leur disponibilité et pour avoir toujours cherché des solutions à mes questions même les plus compliquées. Merci Fabrice, heureusement que tu as été là plus d’une fois.
Merci aux nombreux Novimmuniens qui m’ont toujours encouragée pendant cette thèse et plus précisément au Dr. Nicolas Fischer et à Giovanni Magistrelli. Giovanni, merci pour tes précieux conseils, suggestions et bien sûr pour l’IL6 qui m’a sauvée plusieurs mois de dur labeur ! Les contrôles quoi d’autre!!

Un grand merci à Laetitia, Noémie, Matteo, Domitille, François-Xavier, Vanessa, Virginie L. et tous ceux que j’oublie, pour les nombreux bons moments passés en votre compagnie autour d’une discussion « rapide » dans un couloir ou lors d’un petit repas improvisé dans la petite salle.

Merci également à Carla et Virginie R. Ai-je vraiment besoin de dire pourquoi les filles ? Merci pour votre présence au quotidien, pour toutes nos discussions plus ou moins scientifiques et surtout nos fous rire autour d’un « petit » café ou lors de nos supers lunchs entre copines. Merci d’avoir été et de toujours être là.

Merci à Fanny, Guillemette, Laure, Marlène et Pauline. Merci les filles de m’avoir toujours soutenu, écoutée, encouragée et surtout supportée. C’est bon de vous avoir en amies ! Merci aussi à Mathias, Marc, Maxime J., Maxime T., Mickael et Ondrej de nous avoir laissé papoter entre filles sans regarder le temps, ni juger le niveau de nos discussions!

Je n’oublie pas mes « Mardames », qui se reconnaîtront toutes. Merci les filles pour ces soirées pleines de rires qui m’ont fait tant de bien tout au long de ces années !! A quand la prochaine déjà ?

Plus intimement, je voudrais maintenant remercier mes parents, ma sœur et mon beau-frère qui m’ont suivie dans cette folle aventure de reprise d’études. Vous avez toujours cru en moi et souvent eu beaucoup plus confiance en moi que je ne l’ai eue moi-même. Vous avez toujours été une source de force et de persévérance. Merci de m’avoir supportée et d’avoir si bien pris soin de moi pendant ces années ! Merci également à Nadine, Yvette, Christian et Jean-Claude pour vos encouragements et votre soutien. Je suis heureuse que vous fassiez partie de ma famille. Je pense aussi à mes grands-parents, qui se sont toujours inquiétés de savoir si tout se passait bien pour moi avec mon « stage ».

Enfin, je voudrais dédier cette thèse à mon mari, François et à ma fille, Chloé. Sans toi, François cette thèse n’existerait tout simplement pas. Tu as su être un mari aimant et attentionné comme tu sais si bien l’être et en même temps mon pilier et la force tranquille qui me manquait parfois pour continuer. Merci d’être là. Chloé, ma princesse, merci d’avoir embelli ma dernière année de thèse et toutes celles à venir, tu es ma plus belle réussite.
**Résumé**

Le génome humain comprend 20 325 gènes codant pour des protéines et environ 2 000 n’ont pas encore de fonctions connues. Le groupe CALIPHO a pour objectif d’identifier et de caractériser certaines de ces protéines. Cette thèse s’inscrit dans cette démarche et porte sur la caractérisation d’une protéine mitochondriale de fonction encore inconnue.

Le protéome de la mitochondrie a été estimé à environ 1 500 protéines, dont 1 100 ont été jusqu’à présent identifiées. Cependant, un cinquième de ces protéines n’ont pas encore été caractérisées. La mitochondrie est une organelle complexe qui est impliquée dans de nombreuses fonctions cellulaires dont la production d’énergie via la phosphorylation oxydative. En effet, la membrane interne de la mitochondrie héberge les cinq complexes enzymatiques de la chaîne respiratoire. Il a été récemment prouvé que ces complexes s’associaient entre eux pour former des supercomplexes. Plusieurs groupes proposent des modèles pour l’assemblage de ces complexes et supercomplexes impliquant des facteurs d’assemblage. Cependant, la plupart des études ont été réalisées chez la levure, et peu de choses sont encore connues chez l’Homme. Depuis plusieurs années, de nombreuses mutations sur des gènes nucléaires causant de graves maladies mitochondriales ont été identifiées et associées à des défaillances de la chaîne respiratoire. C’est pourquoi l’identification et la caractérisation fonctionnelle de nouvelles protéines mitochondriales impliquées dans la chaîne respiratoire est un enjeu important.

Le but de cette thèse est de caractériser C11orf83 qui a été identifiée par notre groupe comme une petite protéine potentiellement mitochondriale ayant une fonction importante puisque sa délétion chez la souris est létale. Nous avons tout d’abord confirmé la localisation mitochondriale de C11orf83 et déterminé qu’il s’agissait d’une protéine ancrée dans la membrane interne et que sa partie C-terminale se trouvait dans l’espace intermembranaire. Pour déterminer sa fonction au sein de la mitochondrie, nous avons généré des lignées cellulaires où la synthèse de C11orf83 est inhibée par l’expression stable d’ARN anti-sens. Dans ces cellules, les mitochondries présentent un problème d’ultrastructure associé à une sensibilité accrue à l’apoptose et un changement dans la composition des chaînes latérales d’acide gras des cardiolipines. Nous avons également constaté que l’inhibition de la synthèse de C11orf83 induisait une baisse du niveau d’ATP cellulaire et une faible respiration. Nous avons, plus précisément, mis en évidence un défaut d’assemblage du complexe III de la chaîne respiratoire.

Pour finir, nous avons prouvé qu’en condition de dépolarisation mitochondriale, C11orf83 était coupée par la métalloprotéase OMA1. Des résultats similaires ont été publiés pour la protéine OPA1. En condition de stress, OPA1 est coupée par OMA1, ce qui induit un changement dans l’ultrastructure de la mitochondrie et une fission des mitochondries, pouvant ensuite conduire à l’apoptose. Nous pouvons supposer que la coupure de C11orf83 par OMA1 s’inscrit dans un processus analogue, puisque l’inhibition de C11orf83 perturbe la structure interne de la mitochondrie et augmente la sensibilité de la cellule à l’apoptose.

Une mutation sur la séquence codante du gène C11orf83 (c.59T>A) a été récemment identifiée par Wanschers et al. (2014). Cette mutation induit la synthèse d’une protéine mutée (V20E) instable et non-détectable dans les mitochondries du patient, causant ainsi une déficience du complexe III.

Ainsi, ce travail de thèse en lien avec cette dernière étude clinique permet d’améliorer les connaissances sur l’assemblage du complexe III et la compréhension d’une maladie mitochondriale.
Abstract

The human proteome contains 20,325 proteins among which 2,000 are still not validated at the protein level, nor associated with specific functions. The goal of CALIPHO is to select some of these proteins and to characterize them. This thesis is a part of the CALIPHO project by characterizing a mitochondrial protein of unknown function.

The mammalian mitochondria proteome may contain approximately 1,500 proteins. Around 1,100 mitochondrial proteins have been identified to the date. One-fifth of them still lack functional characterization. Mitochondria are involved in several essential cellular functions, including energy production via the oxidative phosphorylation. The inner membrane hosts the five enzymatic respiratory complexes which are organized in supercomplexes. The formation and assembly processes of these respiratory complexes and supercomplexes require assembly factors and have been widely studied these last years. However, most of works are performed in yeast and the human processes are often inferred through homology and are not based on experimental validations. Mitochondria dysfunctions, and more precisely respiratory chain disorders, underlie a broad spectrum of human diseases. Therefore, the identification and the functional characterization of new mitochondrial proteins represent major challenges in cell biology and in mitochondrial disorder understanding.

In this thesis, we focused on C11orf83, a putative small mitochondrial protein whose knock-out in mice leads to embryonic death. We have shown that C11orf83 is a mitochondrial inner membrane protein facing the intermembrane space. To investigate the function of C11orf83, we engineered HeLa cell clones with undetectable C11orf83 protein levels, using shRNAs. We showed that the depletion of C11orf83 caused a decreased ATP level, an impaired respiration, an abnormal cristae morphology associated to changes in fatty acyl chain composition of cardiolipin, and a higher sensitivity to apoptosis. We also observed a specific defect in the assembly of the bc1 complex of the electron transport chain. More precisely, we demonstrated that C11orf83 is involved in the early stages of assembly of this respiratory complex, by stabilizing the early bc1 core complex (MT-CYB/UQCRB/UQCRQ). As C11orf83 displays some overlapping functions with Cbp4p, a yeast bc1 complex assembly factor, we suggest that C11orf83, now called UQCC3, is the functional human equivalent of Cbp4p. By analogy with this yeast protein, we can suspect that C11orf83 stabilizes MT-CYB in the bc1 core complex. However, we reported some important non-overlapping features
between C11orf83 and the yeast Cbp4p which may reflect the mitochondrial electron transport chain evolutionary differences between *Saccharomyces cerevisiae* and Metazoa. In contrast to Cbp4p which was shown not to interact with the fully assembled bc1 complex and respiratory supercomplexes in yeast, C11orf83 interacts with the bc1 complex and has been detected in the III$_2$/IV supercomplex. Additionally, we observed that the loss of C11orf83 causes a reduction in the amounts of I/III$_2$/IV and III$_2$/IV supercomplexes, with a more pronounced effect for the III$_2$/IV supercomplex. The ability of C11orf83 to bind cardiolipin by its two intermembrane space $\alpha$-helices can be involved in the stabilization of bc1 complex-containing supercomplexes, especially the III$_2$/IV supercomplex.

Finally, we also demonstrated that the OMA1 metalloprotease cleaves C11orf83 in response to mitochondrial depolarization as previously described for OPA1. Indeed, under stress conditions, OMA1 is activated and triggers the inactivation of OPA1 by cleavage leading to mitochondrial fragmentation, to a remodeling of mitochondrial cristae and to the subsequent apoptosis. As we observed that the loss of C11orf83 induced a disorganization of the cristae associated to an enhance sensitivity to apoptosis, the OMA1-mediated cleavage of C11orf83 may also contribute to the mechanism triggering apoptosis under stress.

Wanschers and coll. (2014) identified recently a missense mutation in the coding sequence of *C11orf83* gene (c.59T>A) leading to an unstable V20E mutant protein that becomes undetectable in patient’s mitochondria. This mutation causes a deficiency of the bc1 complex.

Our work combined to this recent study provides new insights into the bc1 complex biogenesis and contributes to a better understanding of a mitochondrial complex III deficiency.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AA</td>
<td>Amino acids</td>
</tr>
<tr>
<td>ADOA</td>
<td>Autosomal dominant optic atrophy</td>
</tr>
<tr>
<td>AGPAT</td>
<td>1-acyl-glycerol-3-phosphate O-acyltransferase</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BN</td>
<td>Blue Native</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CALIPHO</td>
<td>Computational Analysis and Laboratory Investigation of Proteins of Human Origin</td>
</tr>
<tr>
<td>CDP-DAG</td>
<td>Cytidinediphosphate-diacylglycerol</td>
</tr>
<tr>
<td>CGMD</td>
<td>Coarse-grain molecular dynamics simulations</td>
</tr>
<tr>
<td>CL</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>CM</td>
<td>Cristae membrane</td>
</tr>
<tr>
<td>CoQ</td>
<td>Coenzyme Q, ubiquinone</td>
</tr>
<tr>
<td>CoQH2</td>
<td>Reduced coenzyme Q, ubiquinol</td>
</tr>
<tr>
<td>CYC</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>DCI</td>
<td>3,4-dichloroisocoumarin</td>
</tr>
<tr>
<td>DCPIP</td>
<td>2,6-dichlorophenolindophenol sodium salts</td>
</tr>
<tr>
<td>DDM</td>
<td>n-dodecyl-β-D-maltoside</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DTNB</td>
<td>Oxaloacetic acid, 5-5’-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>OXPHOS</td>
<td>Oxidative phosphorylation</td>
</tr>
</tbody>
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Abbreviations (next)

PAGE: Polyacrylamide gel electrophoresis
PC: Phosphatidylcholine
PE: Phosphatidylethanolamine
PG: Phosphatidylglycerol
PG-P: Phosphatidylglycerolphosphate
PI: Phosphatidylinositol
PMSF: Phenylmethanesulfonyl fluoride
POPC: 2-oleoyl-1-pamitoyl-sn-glycero-3-phosphocholine
PS: Phosphatidylserine
RCC1/BLIPII motifs: Regulator of Chromosome Condensation; β-lactamase inhibitor protein-II
RCS: Respiratory supercomplex
ROS: Reactive oxygen species
RT: Room temperature
SDS: Sodium dodecylsulfate
TAP: Tandem affinity purification
TIM: Translocase of the inner membrane
TLC: Thin layer chromatography
TOM: Translocase of the outer membrane
TPCK: N-Tosyl-L-phenylalanine chloromethyl ketone
TRP: Tetraicopeptide repeat
UQCC3: Ubiquinol-cytochrome-c reductase complex assembly factor 3
VDAC: Voltage-dependent anion channel
ΔpH: pH gradient
ΔΨ: Transmembrane potential
2D: Two-dimensional
3D: Three-dimensional
Introduction
I. Mitochondria

Mitochondria are membrane-enclosed organelles the size of bacteria (2-4 μm) found in the cytoplasm of eukaryotic cells and identified for the first time in 1857 by Rudolf Albert von Kölliker (Figure 1 A (Schatz, 2007)). The number of mitochondria per cell varies in a range of a few hundreds to a few thousands according to organism, tissue, cell type, cell-cycle stage and proliferative state. By microscopy analysis, it was estimated that mammalian liver cells contain 1,000 to 2,000 mitochondria corresponding to a fifth of the cell volume, while oocytes are richer in mitochondria with around 100,000 mitochondria (Scheffler, 1999; Alberts et al, 2002). Depending on environmental conditions, cell type and organism, mitochondria present a morphological plasticity. Indeed, thanks to fusion and fission processes mitochondrial shape can vary from a small sphere shape to an interconnected membrane-bound tubular network (van der Bliek et al, 2013).

According to the endosymbiotic theory, mitochondria is descended from formerly free-living α-proteobacteria, which have been engulfed by eukaryotic cell as an endosymbiont around 1.5 billion years ago (Margulis, 1971). This theory explains why mitochondria possess their own DNA (mtDNA). The endosymbiotic relationship resulted in the transfer of many α-proteobacterial genes into the nucleus and the loss of redundant genes (Gray et al, 2001). The mtDNA in vertebrate consists of a double stranded circular DNA molecule of 16.5 kb. It encodes for 22 tRNAs, necessary for mtDNA translation, 2 rRNAs and for 13 proteins corresponding to core subunits of respiratory complexes of the oxidative phosphorylation (OXPHOS) (Anderson et al, 1981; Andrews et al, 1999). In mammals, mitochondria may contain up to 1,500 proteins. Currently, about 1,100 mitochondrial proteins have been confidently identified, but many of them still lack experimental validation (Taylor et al, 2003b, 2003a; Pagliarini et al, 2008; Meisinger et al, 2008). As the mammalian mitochondrial genome only encodes for a few proteins, the great majority of the mitoproteome are proteins encoded by the nuclear genome, translated by cytosolic ribosomes and imported into mitochondria through complex mechanisms (Schmidt et al, 2010).
I.1. Mitochondria functions

Mitochondria are required for cell growth due to their numerous and vast range of functions. Among their functions, these organelles, well-known as “the power house of the cell”, carry out the OXPHOS that combines electron transport with cell respiration and adenosine triphosphate (ATP) synthesis to provide most of the energy used by the cells (Gilkerson et al, 2003). The electron transport chain (ETC) formed by respiratory complexes generates a transmembrane electrochemical potential which consists both in a pH gradient (ΔpH) and an electrical gradient (ΔΨ). The proton-motive force generated by ETC is used by the ATP synthase to produce ATP. OXPHOS complexes hosted by mitochondria will be more discussed in the next section.

In addition to OXPHOS, mitochondria are involved in the regulation of apoptotic cell death (Newmeyer & Ferguson-Miller, 2003). Apoptosis is both required as a homeostatic mechanism (embryonic development, maintenance of normal tissue homeostasis and aging) and as a defense process (immune reactions, diseases and toxicant-mediated apoptosis) (Elmore, 2007). Apoptosis can be elicited by intracellular stimuli such as metabolic imbalance, cell cycle perturbation, and DNA damages or extracellular factors as inflammatory mediators (Elmore, 2007). Mitochondria play a central role in this process by the release of mitochondrial proteins such as cytochrome c (CYC), which initiates an intracellular caspase proteolytic cascade (Martinou et al, 2000). This cascade leads to the cleavage of substrates like the poly(ADP-ribose) polymerase, which becomes unable to respond to DNA strand breaks (Boulares et al, 1999), or the nuclear lamins, inducing an irreversible breakdown of the nuclear matrix (Dynlacht et al, 1999). During apoptosis, the release of other pro-apoptotic proteins such as DIABLO or apoptosis-inducing factor (AIF) is also promoted (Zhou et al, 2005; Candé et al, 2002). All these processes trigger the controlled death of the cell.

Mitochondria also hosts Krebs cycle enzymes and take part in iron–sulfur cluster generation (Lill & Mühlenhoff, 2005), lipid metabolism pathways (Tatsuta et al, 2014), calcium signaling (Graier et al, 2007) and reactive oxygen species generation (ROS) (Gilkerson et al, 2003).
I.2. Mitochondrial structure

Mitochondria are surrounded by two specialized membranes, the outer membrane (OM) and the inner membrane (IM), which define two internal compartments: the intermembrane space (IMS) which has soluble specific proteins like pro-apoptotic proteins (CYC, DIABLO and AIF) and the internal matrix which hosts the Krebs cycle enzymes and contains mtDNA (Figure 1 A and B).

I.2.1. Outer membrane

OM allows communication between mitochondria and the rest of the cell, by exchanging metabolites, adenine nucleotides and ions. Mitochondrial voltage-dependent anion channels (VDACs), also called porins, are the most abundant integral membrane proteins found in the OM which form pores that are permeable to small molecules (Lindén et al, 1984; Colombini, 1979). These channels have been involved in apoptosis by binding to proteins of the Bcl-2 family and by regulating the permeabilization of the OM. However the molecular mechanisms used are still under debate (Kroemer et al, 2007; Shi et al, 2003). The OM also corresponds to the entry gate of the mitochondrial proteins synthesized in the cytosol. Import of these proteins requires a signaling sequence at their N-terminus recognized by the translocase of the OM (TOM) complex whose main components are TOMM20 and TOMM70A (Figure 1 B). Proteins imported actively cross the OM to subsequently reach one of the mitochondrial sub-compartments (Wiedemann et al, 2004).

I.2.2. Inner boundary membrane

In combination with the matrix, the IM represents the major working part of the mitochondria. To this purpose, the IM present a protein-lipid ratio higher than that of the OM (Haller-mayer & Neupert, 1974). The IM is organized in two domains, (1) the inner boundary membrane (IBM) which is closely juxtaposed to the OM and (2) the cristae membrane (CM). The boundary membrane forming with the OM the double-layered envelope of the mitochondria is enriched in protein complexes involved in the import of nuclear encoded preproteins. In this region, the TOM complex cooperates with the two translocase IM complexes (TIM) (Figure 1 B). The TIM22 complex, containing TIMM10 for example, allows the import of integral mitochondrial
carrier proteins and the TIM23 complex, including TIMM44, is used by preproteins having matrix-targeting signal (Bauer et al., 2000).

1.2.3. Cristae and proteins and lipids involved in their maintenance

The cristae membrane corresponds to invaginations of the inner boundary membrane that protrude into the matrix space. Cristae are connected to the inner boundary membrane by cristae junctions. Cristae junctions are narrow tubular-like structures with a diameter of around 28 nm and an average length of 30–40 nm (Frey & Mannella, 2000; Frey et al., 2002). Tubular cristae can merge with each other leading to larger cristae with varying lengths. As respiratory complexes are found enriched in cristae, these structures have been defined as the site of OXPHOS (Gilkerson et al., 2003; Kay et al., 1985; Wilkens et al., 2012). Moreover, cristae junctions may act as a diffusion barrier for specific metabolites like protons or ADP during respiration. The cristae structure permits an optimization of the mitochondrial traffic of adenylates by minimizing their path between translocation sites (translocase/ANT sites) and metabolic sites (ATPase sites) (Demongeot et al., 2007; Mannella et al., 2001). Additionally, cristae junctions allow the compartmentalization of IMS proteins, like CYC that is sequestered in the intracristal space. Indeed, it was shown that approximatively 85% of the total CYC is stored in the cristae and a remodeling of cristae is required at early stages of apoptosis to facilitate CYC release from the IMS (Figure 1 B) (Scorrano et al., 2002). The cristae morphology is important for mitochondrial functions and the following section details the protein and lipids involved in its maintenance (Figure 6).
I.2.3.1 The mammalian MICOS complex

The mitochondrial contact site complex (MICOS (Hoppins et al., 2011)), also called mitochondrial inner membrane organizing system (MINOS (von der Malsburg et al., 2011)) or mitochondrial organizing structure (MitOS (Harner et al., 2011)) is a large protein complex of around 700 kDa conserved from yeast to mammals involved in mitochondrial ultrastructure maintenance. In 2014, the nomenclature for this complex was unified and “mitochondrial contact site and cristae organizing system” (MICOS) chosen as official name. The MICOS subunits are termed in any organism, MICn with the number ‘n’ corresponding of the molecular mass of the identified mature protein in kDa (Pfanner et al., 2014). MICOS plays a central role in IM architecture maintenance, cristae formation and IM/OM contact sites formation. This complex is preferentially located close to cristae junctions. Currently, seven proteins are identified for the yeast complex, and six for the mammalian complex: MIC60/Mitofilin (John et al., 2005), MIC10/MINOS1 (Alkhaja et al., 2012), MIC19/CHCHD3 (Darshi et al., 2011), MIC25/CHCM1/CHCHD6 (An et al., 2012), MIC27/APOOL and MIC26/APOO (Weber et al., 2013).
The protein QIL1 has been recently identified as a new subunit of the mammalian MICOS complex required for the assembly of this complex (Guarani et al., 2015). Downregulation of several MICOS proteins in cells of both organisms dramatically alters the mitochondrial ultrastructure, with formation of large concentric stacks of cristae membrane and impacts the presence of cristae junctions to different degrees (Hoppins et al., 2011; Rabl et al., 2009; Harner et al., 2011; von der Malsburg et al., 2011; John et al., 2005; An et al., 2012; Alkhaja et al., 2012; Weber et al., 2013; Darshi et al., 2011). In addition, the MICOS complex has close interaction with some OM proteins such as SAMM50 (Ott et al., 2012) and the chaperone-like protein DNAJC11 (Alkhaja et al., 2012). The downregulation of these OM proteins impairs the cristae structure, suggesting the OM is involved in the cristae morphology maintenance via the MICOS complex (Ott et al., 2012; Ioakeimidis et al., 2014). However, the molecular mechanism responsible for mitochondrial cristae architecture maintenance via the MICOS complex remains unclear.

**I.2.3.2 OPA1 and mitochondrial proteases**

The dynamin-like GTPase Mgm1/OPA1 is a IM protein facing IMS which regulates mitochondria fusion (Cipolat et al., 2004) and is associated with mutations causing autosomal dominant optic atrophy (ADOA) (Delettre et al., 2000; Alexander et al., 2000). Patient-derived skin fibroblasts with heterozygous OPA1 mutations present mitochondria with ultrastructure alterations (Agier et al., 2012). Similar observations were obtained with OPA1 downregulation experiments; OPA1-deficient cells have severe defects of cristae organization (irregular shape of cristae membranes, large cristae junctions) and present an accelerated CYC release under apoptosis conditions (Arnoult et al., 2005; Frezza et al., 2006; Olichon et al., 2003; Ishihara et al., 2006; Olichon et al., 2002). OPA1 is localized at cristae junction and forms oligomers of high molecular weight which seem to be involved in maintaining cristae junctions (Frezza et al., 2006; Satoh et al., 2003; Cipolat et al., 2006).

Through alternative splicing the OPA1 gene can produce eight transcripts which are expressed in a tissue-dependent manner (Delettre et al., 2001). Each splice variant is translated into a long preprotein that is cleaved by mitochondrial processing peptidase (MPP) to generate the long, mature isoforms (L-OPA1) (**Figure 2 A**). In the literature, only two bands (called a and b) were reported for all L-OPA1 isoforms on gel electrophoresis, probably corresponding to a mixture of the different splice forms (**Figure 2 B**) (Song et al., 2007). All L-OPA1 isoforms display
a cleavage site named S1, and the L-OPA1a isoforms have an additional one named S2. Processing at these sites can produce three different short isoforms (S-OPA1c, d and e) that all lack the transmembrane domain (Figure 2 A and B). (Delettre et al., 2001; Song et al., 2007). There are evidences that L-OPA1 isoforms are sufficient to mediate complete fusion of mitochondria and maintain cristae organization, whereas the accumulation of S-OPA1 isoforms may trigger mitochondrial fission, either by inhibiting fusion or by stimulating fission (Anand et al., 2014; Ishihara et al., 2006).

**Figure 2: OPA1 Isoforms**

A) Schematic representation of the eight OPA1 splice variants and the long and short OPA1 isoforms which can be generated. Splice variants differ in the presence or absence of exons 4, 4b, and 5b. The cleavage of the N-terminal mitochondrial targeting sequence (MTS) by mitochondrial processing peptidase (MPP) generates the long isoforms (L-OPA1). Two classes of L-OPA1 can be generated, the isoforms a displaying the two cleavage sites (S1 and S2 showed in red and orange, respectively) and the isoforms b presenting only the site S1. Proteolytic cleavage at sites S1 or S2 produces the three short isoforms (c,d and e) lacking the transmembrane (TM) domain. Adapted from (Song et al., 2007) B) OPA1 was detected on human osteosarcoma 143B cell line by western blot analysis using an anti-OPA1 antibody. The five OPA1 isoforms detected are indicated. Data from (Ehses et al., 2009)

Several proteases were proposed to trigger the proteolysis leading to the formation of S-OPA1 forms (Ishihara et al., 2006; Griparic et al., 2007; Ehses et al., 2009; Head et al., 2009; Kieper et al., 2010; Cipolat et al., 2006; Anand et al., 2014). The i-AAA protease YME1L, a membrane-bound ATP-dependent peptidase with its catalytic site exposed to the IMS, was proposed for the constitutive processing at the cleavage site S2 (Song et al., 2007; Griparic et al., 2007). Recent data confirmed this YME1L-constitutive OPA1 cleavage and its involvement in the
mitochondria morphology and cristae structure regulation (Figure 3 A) (Anand et al, 2014; Stiburek et al, 2012). The rhomboid protease PARL (Cipolat et al, 2006; Frezza et al, 2006) and the m-AAA proteases SPG7 and AFG3L2 (Ehses et al, 2009; Duvezin-Caubet et al, 2007) were proposed for the constitutive processing of L-OPA1 at S1. However, in contrast with results obtained in yeast, the downregulation of these three proteases in mouse embryonic fibroblasts (MEF) or in HeLa cells did not result in accumulation of the L-OPA1 isoforms (Duvezin-Caubet et al, 2007; Ishihara et al, 2006). In 2009, the Langer’s group proposed OMA1, a mitochondrial metalloprotease as the protease responsible of the constitutive OPA1 cleavage at the S1 site when m-AAA proteases are impaired (Ehses et al, 2009). OMA1-deficient MEF lacked S-OPA1 isoforms generated by the cleavage at S1 site, confirming the sole involvement of OMA1 in constitutive OPA1 cleavage at this site (Figure 3 A) (Anand et al, 2014; Baker et al, 2014; Quirós et al, 2012). The OMA1-mediated cleavage of OPA1 at S1 can be accelerated under stress conditions such as loss of ΔΨ, ATP deficiency, or apoptotic stimuli (Figure 3 B). This stress-induced cleavage leads to the accumulation of S-OPA1 isoforms and promotes mitochondrial fragmentation (Ehses et al, 2009; Head et al, 2009; Quirós et al, 2012; Baker et al, 2014; Zhang et al, 2014; Baricault et al, 2007; Jiang et al, 2014). Accordingly, OMA1-deficient cells conserve L-OPA1 isoforms under stress condition, maintain a mitochondrial tubular morphology and are less prone to apoptosis (Ehses et al, 2009; Head et al, 2009; Baker et al, 2014; Anand et al, 2014).

OMA1 is a zinc metalloprotease of the M48 family, located in the IM with its C-terminal domain in IMS (Baker et al, 2014). It was first identified in yeast as a protease having an overlapping activity with m-AAA proteases and involved in mitochondrial quality control (Kaser et al, 2003). OMA1 is synthetized as precursor protein of 60 kDa, imported and cleaved to obtain a mature IM protein of approximatively 43 kDa (Baker et al, 2014). Zhang and coll. showed that under stress stimuli, the mature OMA1 is self-cleaved at its C-terminus to generate a protein of 35 kDa lacking residues 443 to 452 (Zhang et al, 2014). This 35 kDa–OMA1 form seems to be sufficient to process OPA1 upon ΔΨ loss or apoptosis (Figure 3 B) (Zhang et al, 2014). However, the 35kDa–OMA1 form is less stable than the mature 43 kDa-OMA1 and promotes its own degradation (Zhang et al, 2014). The autoproteolysis of active OMA1 may contribute to control the stress response by preventing OPA1 processing and subsequent mitochondrial
fragmentation (Baker et al, 2014). At the N-terminal matrix-exposed portion of 43 kDa-OMA1 a positively charged region following a hydrophobic stretch has been identified and seems required for OMA1 activation under stress condition (Figure 3 B) (Baker et al, 2014). Baker and colleagues postulated that this positively charged amino acids (aa) cluster would act as a domain sensor involved in ionic interaction of OMA1 with the negative head of phospholipids. Under stress conditions, this domain could trigger a conformational change of the 43 kDa-OMA1 form, inducing its activation and C-terminus self-cleavage (Baker et al, 2014).

On the other hand, the prohibitin complex which is a ring-like complex formed by several PHB1 and PHB2 proteins, seems to regulate cristae morphology via the control of OPA1 processing. The knockdown of PHB1 or PHB2 leads to selective loss of larger OPA1 isoforms inducing an aberrant mitochondrial ultrastructure and an increase of cell sensitivity towards apoptotic stimuli (Zhang et al, 2014; Merkwirth et al, 2008; Song et al, 2009). Recently, Zhang and coll. observed that PHB1 or PHB2 knockdown resulted in a clear decrease of the 43 kDa-OMA1 form and an increase of the active 35 kDa-OMA1 form under depolarizing conditions. These results suggest that the prohibitin complex may be involved in OPA1 processing and cristae morphology regulation, by stabilizing the mature 43 kDa-OMA1 form (Figure 3 B) (Zhang et al, 2014).

Finally, the study of Oma1-mutant mice has provided the first in vivo evidence of the importance of OMA1 to maintain metabolic homeostasis. Oma1-mutant mice exhibit an increase in body weight with metabolic alterations, reduced energy expenditure and altered thermogenic regulation (Quirós et al, 2012). Identification of new OMA1 substrates is therefore important to better understand the molecular mechanism of OMA1 involvement in mitochondrial ultrastructure regulation and metabolic homeostasis maintenance.
Introduction

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Figure 3: Pathways for OPA1 proteolytic processing

A) In healthy mitochondria, the constitutive cleavage pathway allows conversion of a fraction of L-OPA1 isoforms (a and b) into S-OPA1 isoforms (c, d and e). OMA1 cleaves L-OPA1 at the S1 site, whereas the processing at the S2 site is performed by YME1L. The active form of OMA1 which undergoes this constitutive cleavage is still unknown: Baker and colleagues proposed the mature form of 43 kDa (Baker et al., 2014). B) Under stress conditions, (e.g. dissipation of ΔΨ, ATP deficiency or apoptosis), all L-OPA1 are converted into S-OPA1 by OMA1-mediated cleavage at S1. Stress conditions lead to an activation of the 43 kDa-OMA1 mature form by a self-cleavage at its C-terminus generating the active 35 kDa-OMA1 form. The cleavage site is indicated by a red dot and arrow on the IMS-part of OMA1. The newly produced active OMA1 is more unstable than the other OMA1 forms, so after having cleaved L-OPA1, it undergoes autoproteolysis. How OMA1 detects the stress to undergo activation is still undiscovered; The positively charged aa cluster at the N-terminal part of OMA1 (representing by three “+”) has been proposed as a domain sensor (Baker et al., 2014). In addition, data reported by Zhang and colleagues suggested that the presence of a prohibitin (PHB) complex could stabilize the 43 kDa–OMA1 form (Zhang et al., 2014).

I.2.3.3 Cardiolipins

In addition to proteins, phospholipids are important to maintain membrane structural integrity. Mitochondrial membranes have the common classes of phospholipids usually identified in cell membranes (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidic acid (PA)), but also the mitochondrial-specific phospholipid cardiolipin (CL). CL or 1,3-bis(sn-3'-phosphatidyl)-sn-glycerol is a dimeric phospholipid with two glycerophosphate backbones, four fatty acyl chains and is hosted predominantly in the IM. In rat liver mitochondria, CL respectively contributes to around 18 % of IM and less than 1 % of OM total phospholipids (Horvath & Daum, 2013).

CL is synthesized in the IM by an enzymatic cascade (Figure 4) (Kent, 1995; Lu & Claypool, 2015). An acyl-glycerol-3-phosphate O-acyltransferase (AGPAT) converts a glycerol-3-phosphate to PA by a double acylation. The PA molecule is processed by the phosphatidate cytidylyltransferase (TAMM41) to form an intermediate cytidinediphosphate-diacylglycerol (CDP-
DAG). Then, the enzyme phosphatidylglycerolphosphate (PG-P) synthase (PGS1) converts CDP-DAG into PGP which is dephosphorylated by the IM tyrosine phosphatase protein 1 (PTPMT1) to obtain a PG. The last step of CL synthesis is the condensation of CDP-DG with PG by CL synthase (CRLS1).

The fatty acid chains of CL can vary in length and unsaturation and their composition differs between cell types and tissues within the same organism. For example human heart mitochondria are enriched in CL with linoleyl chains (18:2), whereas lymphoblasts predominantly have tetraoleyl-CL (C18:1) (Schlame et al, 2005). To obtain these diverse specific fatty acyl chains compositions, newly synthetized CL undergo a remodeling process which can occur by two mechanisms (Ye et al, 2014). The first mechanism is a deacylation-reacylation cycle, in which a phospholipase A2 (PLA2G6, (Malhotra et al, 2009)) deacylates the nascent CL to generate a monolysocardiolipin (MLCL). This intermediate is reacylated by the transacylase tafazzin TAZ (Houtkooper et al, 2009b) or by monolyso-cardiolipin acyltransferase (HADHA/MLCLAT (Taylor & Hatch, 2003)) or acyl-CoA:lysocardioplin acyltransferase (LCLAT1/ALCAT (Cao et al, 2004)) to obtain a mature CL. The second mechanism is performed by TAZ and corresponds to a direct transacylation between a phospholipid and the immature CL (Ye et al, 2014; Xu et al, 2003).

**Figure 4: Biosynthesis and remodeling of CL.** Phosphatidic acid is formed from glycerol-3-phosphate by glycerol-3-phosphate acyltransferase (AGPAT). The phosphatidate cytidylyltransferase (TAMM41) uses the PA molecule to generate a molecule of cytidinediphosphate-diacylglycerol (CDP-DAG) which is converted into phosphatidylglycerolphosphate (PG-P) thanks to the phosphatidylglycerolphosphate synthase synthase (PGS1). PG-P is dephosphorylated by the tyrosine phosphatase protein 1 (PTPMT1) to obtain PG. An immature CL is formed from PG by CL synthase (CRLS1). The newly synthetized CL undergoes a remodeling process. The CL can be deacetylated by a phospholipase A2 (PLA2G6) to generate a monolysocardiolipin (MLCL) which can be reacylated by tafazzin (TAZ), monolyso-cardiolipin acyltransferase (HADHA) or acyl-CoA:lysocardioplin acyltransferase (LCLAT1) in order to obtain mature CL. Alternatively, a direct transacylation, using the transacylase TAZ, can occur between a phospholipid and immature CL to generate mature CL.
With four acyl chains and a large number of possible fatty acids (14 in human), CL can potentially be formed of a huge number of molecular species. However, *in vivo* the remodeling process generates structural uniformity among CL and a high proportion of CL with two identical phosphatidyl residues (Schlame *et al*, 2005; Schlame & Ren, 2009). CL have been involved in diverse mitochondria functions by their interaction with several proteins, such as CYC (Kagan *et al*, 2009) or respiratory chain complexes (Arnarez *et al*, 2013a, 2013b; Palsdottir & Hunte, 2004). CL, as all phospholipids, are important to maintain the structural integrity of membranes. Because CL have a high intrinsic curvature, it was suggested that the CL microdomains identified at the contact sites between IM and OM (Sorice *et al*, 2009) could stabilize the geometry of curved regions of the membrane (Renner & Weibel, 2011; Schlame & Ren, 2009). The observation of CL clusters at the negatively curved regions in bacteria supports this hypothesis (Renner & Weibel, 2011). The importance of CL and their remodeling in cristae morphology is underlined in Barth syndrome, a X-linked recessive disorder, caused by mutations in the TAZ gene (Schlame & Ren, 2006). Lymphoblast mitochondria of Barth syndrome patients have a complete reorganization of the fatty acid profile of CL associated to a disorganized cristae (Acehan *et al*, 2007; Schlame *et al*, 2003). In accordance with these data, CL deficiencies induced by downregulation of CRLS1 or PGS1 in mammalian cells lead to an altered ultrastructure (Ohtsuka *et al*, 1993; Choi *et al*, 2007). Beside the IM structural function of CL linked to their molecular shape, CL seem also involved in the regulation of cristae morphology by promoting the assembly of ATP synthase dimers (Acehan *et al*, 2011).

### 1.2.3.4 ATP synthase

ATP synthase (F<sub>1</sub>F<sub>0</sub> ATPase, EC 3.6.3.14) or Complex V (CV), is an enzyme that has dual functions in energy production and cristae structure. This enzyme is composed by a hydrophilic catalytic unit (F<sub>1</sub>) located in the matrix, and a hydrophobic F<sub>0</sub>-part anchored in the IM (Fillingame, 1999). It has been shown that ATP synthase forms dimers organized like ribbons at the apex of cristae ([Figure 6](#)) (Davies *et al*, 2012; Dudkina *et al*, 2005b; Strauss *et al*, 2008). Electron tomography of intact mitochondria and cristae membranes from various species shows that the ATP synthase dimer has a V-shaped structure with an angle of around 90°C between monomers, which induces bending of the IM ([Figure 5](# A and B)) (Minauro-Sanmiguel *et al*, 2005; Davies *et al*, 2011; Baker *et al*, 2012). The ATP synthase dimers are assembled in rows which
may lead to the formation of the curved cristae ridges (Figure 5 C) (Strauss et al, 2008; Minauro-Sanmiguel et al, 2005; Paumard et al, 2002; Davies et al, 2011; Baker et al, 2012; Dudkina et al, 2005a; Davies et al, 2012). The role of ATP synthase on cristae morphology was confirmed by genetic disruption in yeast. Mutants with impaired dimer ATP synthase show extensive changes in cristae morphology with cristae membrane forming onion-like structures (Arselin et al, 2003; Paumard et al, 2002; Gavin et al, 2004; Davies et al, 2012).

Figure 5: Membrane curvature induced by ATP synthase dimers. A) Model of the V-shape formed by the dimers of ATP synthase proposed by (Baker et al, 2012). In this model, the angle between the two monomers in the dimer would be ~86°, which is in accordance with the ~90° observed by electron tomography (Minauro-Sanmiguel et al, 2005; Davies et al, 2011; Baker et al, 2012). B) Perspective views of ATP synthase dimers alone or C) assembled in rows, inducing a curvature of the simulated membrane. Data from (Davies et al, 2012).

As depicted in figure 6, the IM components described above (MICOS complex, OPA1, CL and ATP synthase) contribute all together in the formation and maintenance of the cristae
II. Electron transport chain (ETC)

The ETC, also called respiratory chain, is composed by four transmembrane complexes (Figure 7): Complex I (CI, NADH:ubiquinone oxidoreductase, EC 1.6.5.3), Complex II (CII, succinate:ubiquinone oxidoreductase, EC 1.3.5.1), Complex III (CIII, ubiquinol:ferricytochrome c oxidoreductase, EC 1.10.2.2), and Complex IV (CIV, cytochrome c oxidoreductase, EC 1.9.3.1). Succinate, nicotinamide-adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide (FADH$_2$) generated by glycolysis, fatty acid oxidation and the Krebs cycle are substrates of ETC. Electrons released during the oxidation of NADH by CI, or by oxidation of FADH$_2$ by CII, are transferred to ubiquinone (coenzyme Q, CoQ). CIII, also called bc1 complex, transfers electrons from reduced ubiquinone to the carrier, CYC. Within CIV, electrons released by oxidation of the reduced cytochrome are transferred to O$_2$, the final electron acceptor, leading to H$_2$O. The electron flow through the ETC is associated with transport of protons from matrix to IMS, generating a proton gradient across the IM. Then, the ATP synthase or CV uses the proton-
motive force induced by the respiration to generate ATP from ADP and P$_i$ (Figure 7) (Saraste, 1999; Lodish et al, 2000). The OXPHOS complexes in IM are present in non-equal amounts. In bovine heart mitochondria, for each CI, there are about one CII, three CIII/bc1 complexes, seven CIV and three F$_i$F$_0$ ATPases (Hatefi, 1985; Schagger & Pfeiffer, 2001).

**Figure 7: Schematic representation of the electron transport chain (ETC) and the ATP synthase (OXPHOS system).** The electron pathways between complexes and mobile carriers, Cytochrome c (CYC) and Coenzyme Q (CoQ) are represented by blue arrows. Respiratory complexes are colored in blue (CI), red (CII), yellow (CIII, bc1 complex), purple (CIV) and green (CV, ATP synthase). CoQ is represented by a small red dot and CYC as a blue-filled triangle.

OXPHOS complexes are composed by more than 80 polypeptides with only 13 polypeptides encoded by mtDNA (CI: ND1, 2, 3, 4, 4L, 5, 6, CIII: MT-CYB, CIV:COI, II, III and CV: ATPase 6 and 8). Their formation and assembly are elaborated mechanisms which require additional nuclear-encoded proteins called assembly factors (Ghezzi & Zeviani, 2012). Assembly factors have been reported for all OXPHOS complexes and their physiological importance is evidenced by the number of human diseases associated with mutations in genes encoding them (Ghezzi & Zeviani, 2012). For example, more than 30 cytochrome c oxidase assembly factors were identified by analysis of human patients with CIV deficiencies and respiratory deficient yeast cells (Khalimonchuk & Rödel, 2005; Soto et al, 2012). Identification and characterization of new assembly factors give important clues to understand the biogenesis of the mitochondrial respiratory chain. Recently, MCUR1/CCDC90A and COA6/C1orf31 were identified as CIV assembly factors which respectively stabilize newly synthesized COXII and participate to the copper delivery pathway essential for assembly and stability of CIV (Paupe et al, 2015; Baertling et al, 2015; Ghosh et al, 2014). Factors involved in bc1 complex assembly are described later.
III. Ubiquinol-cytochrome c reductase

Complex III, or bc1 complex, is a central component of the ETC and is well-conserved among species (Berry et al., 2000). Bacterial bc1 complexes are found in the plasma membrane of both gram-negative and gram-positive bacteria (Trumpower, 1990). The thylakoid membranes of cyanobacteria and chloroplasts of higher plants host a structural and functional analogue of the bc1 complex, called cytochrome b6f complex (Baniulis et al., 2008).

III.1. The catalytic mechanism of the bc1 complex: the Q-cycle

The bc1 complex transfers electrons from the reduced lipid-soluble coenzyme Q (CoQH2) to the water-soluble IMS protein, CYC. The mechanism called Q-cycle, first proposed in 1976 (Mitchell, 1976), couples the electron flow to proton translocation across the membrane (Figure 8). The catalytic core of the mammalian bc1 complex is composed of three redox subunits (cytochrome b (MT-CYB), containing hemes, bL and bH, cytochrome c1 (CYC1), with a heme c1 and the Rieske protein (UQCRFS1), having a [2Fe–2S] iron–sulfur cluster) and two quinone processing sites (Robertson et al., 1993). The Qo (out) site, also called the Qp (positive) site, is localized to the IMS side of the IM and is the ubiquinol oxidation site. The Qi (in) site or Qn (negative) site faces the matrix and hosts all quinone reductions.

The reduced coenzyme CoQH2 diffuses in the membrane from CI to the Qo site of the bc1 complex. CoQH2 gives one electron which is transferred to the Rieske protein then to CYC1 and finally to the final acceptor, CYC. This process is coupled with the release of two protons from CoQH2 to the IMS and generates a semiquinone radical (CoQ-). This intermediate ubiquinone releases an electron to heme bL in order to generate a fully oxidized coenzyme Q (CoQ). This second electron crosses the IM via heme bH which is localized at the matrix side of the IM, and reaches a second molecule of oxidized ubiquinone (CoQ) bound to Qi-site which then becomes a semiquinone radical (CoQ-) (Figure 8, left). In a second time (Figure 8, right), the Qo-site welcomes a second molecule of reduced coenzyme CoQH2 which releases its two electrons as previously explained for the first round. One electron is collected by the Rieske protein to reach CYC via heme c1 and the second electron is caught by heme bL. This last electron gets to the Qi-site, via heme bH, to reduce the semiquinone radical (CoQ-) generated.
during the first round. To fully reduce this intermediate, two protons from the matrix are picked up. The new formed CoQH2 molecule moves out to the Qi-site to return to the membrane pool. In summary, only one molecule of ubiquinol (CoQH2) is fully oxidized in order to reduce two molecules of CYC. As only two protons are collected from the matrix despite the four protons released in the IMS, the Q cycle generates a proton gradient (Crofts et al., 2008; Crofts, 2004; Lodish et al., 2000).

**Figure 8: Q-cycle mechanism.** The electron transfer within the bc1 complex occurs thanks to the Q-cycle which can be divided in two parts. First, a molecule of reduced coenzyme CoQH2 binds at the Qo-site of the complex and gives two electrons. One reaches the iron-sulfur cluster [2Fe–2S] of Rieske protein (UQCRFS1) to be transferred to the heme c1 of CYC1 and finally to the mobile carrier CYC. The second electron is taken by the heme b₃, then by the heme b₅ of MT-CYB until it reaches the Qi-site of the complex where an oxidized ubiquinone (CoQ) is waiting for it. The oxidation of the CoQH2 at the Qo-site leads to the release of two protons to the IMS. During the second part of this Q-cycle, a new molecule of CoQH2 binds at the Qo-site and delivers once again its two protons and its two electrons which undergo the same pathway described in the first part. At the Qi-site the semi-quinone radical (CoQ•-) formed during the first round is fully reduced thanks to the two protons picked up from the matrix and the second electron given by the heme b₅. In summary, two CoQH2 molecules are oxidized at the Qo-site to reduce one CoQ molecule and two CYC.
III.2. Structure of the bc1 complex

The mammalian bc1 complex exists in the IM as a symmetrical homodimer (Schägger, 2001). Each monomer has a molecular weight of 240 kDa and is composed by 11 subunits among which only MT-CYB is encoded by the mitochondrial genome (Iwata et al., 1998; Schägger et al., 1986). Despite the presence of an additional subunit in the mammalian bc1 complex, corresponding to the cleaved presequence of the Rieske protein, the structure of bc1 complex is similar in yeast and mammals (Table 1) (Iwata et al., 1998; Lange & Hunte, 2002).

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<td>Cor2p</td>
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<td>Cobp/Cytochrome b</td>
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<td>Cyc1p/Cytochrome c1</td>
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<td>Rip1p/Rieske protein</td>
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Table 1: Mammalian bc1 complex subunits and their yeast homologs

Several crystal structures of both yeast and bovine bc1 complexes have been published and provide valuable information on bc1 complex subunits and interactions between them (Figure 9) (Iwata et al., 1998; Berry et al., 2000; Palsdottir et al., 2003; Lange & Hunte, 2002; Akiba et al., 1996).
III.2.1. The bc1 catalytic core subunits

The three essential subunits forming the catalytic core (MT-CYB, CYC1, and UQCRFS1) are highly conserved. The eight transmembrane helices of the MT-CYB subunit of each monomer form the center of the complex. CYC1 and UQCRFS1 are small integral proteins anchored in the IM by their single transmembrane domains. Their prosthetic groups (heme c1 and 2Fe–2S cluster, respectively) are localized in the IMS (Iwata et al., 1998). In contrast to CYC1 which has a rigid globular domain, structural analyses and biochemistry experiments highlighted a flexible extrinsic domain for UQCRFS1 (called ED-UQCRFS1) required for bc1 function (Iwata et al., 1998; Xiao et al., 2000; Ghosh et al., 2001). The ED-UQCRFS1 can move between three positional states: the c1- and b-position states where the [2Fe-2S] cluster is close to heme c1 or bL, respectively and an intermediate position ("int"), with ED-UQCRFS1 localized between the c1- and b- sites. In absence of substrate, ED-UQCRFS1 displays an int position. The binding of a CoQH2 substrate to the Qo pocket leads to an ED-UQCRFS1 conformational change from int state to the b-position. A transient complex MT-CYB/CoQH2/ED-UQCRFS1 is generated and CoQH2 is oxidized by transferring one electron to heme bL and another to the [2Fe-2S] cluster. The accepted model proposes that the electron transfer from heme bL to bH facilitates the new conformation switch of ED-UQCRFS1 at the c1-position, bringing closer the [2Fe-2S] cluster.
and heme c1 and allowing the second electron transfer (Xiao et al, 2000; Xia et al, 2013; Yu et al, 2008; Iwata et al, 1998).

### III.2.2. The bc1 complex supernumerary subunits

In contrast with these catalytic subunits, all other bc1 complex subunits are poorly conserved non-redox polypeptides. The bc1 complexes of *Paratrococcus denitrificans* (Trumpower, 1991) *Rhodopseudomonas rubrum* (Kriauciu纳斯 et al, 1989), and *Rhodobacter capsulatus* (Robertson et al, 1993), have no supernumerary subunits. In other species, the number of these subunits varies: the *Rhodobacter sphaeroides* bc1 complex contains only one supernumerary subunit (Yu et al, 1999), while the yeast and bovine bc1 complexes have seven and eight supernumerary subunits, respectively (Schägger et al, 1986; Ljungdahl et al, 1987). The function of these subunits has not yet been established, but as bc1 complexes without supernumerary subunits are less stable, their role in the bc1 complex stability is currently assumed (Schägger et al, 1986). Among these supernumerary subunits, two large extrinsic core proteins are found on the matrix side of the complex, UQCR1/Cor1p (SU1) and UQCR2/Cor2p (SU2). The core proteins share 21% of sequence identity and a similar three-dimensional (3D) structure (Xia et al, 1997). The NH2-terminal domain of each protein interacts with the COOH-terminal domain of the other generating a cavity which can be visualized by EM (Akiba et al, 1996). In the bovine bc1 complex, the subunit 9, corresponding to the 78-aa mitochondrial targeting pre-sequence of UQCRFS1, is localized in this cavity and mostly interacts with UQCR2 (Iwata et al, 1998; Brandt et al, 1993). Plant purified bc1 complexes exhibit, in addition to the electron transfer activity, a mitochondrial processing peptidase (MPP) activity supported by the bc1 complex and more precisely by core proteins (Glaser et al, 1994). The mammalian core proteins are homologous to the α and β subunits of MPP (Xia et al, 1997) and they can display MPP activity in conditions where the electron transfer activity of the bc1 complex is inhibited (Deng et al, 1998).

UQCRB/Qcr7p (SU6) is a small protein localized on the matrix side of IM which make contact with UQCR1/cor1p and UQCR2/cor2p. UQCRH/Qcr6p (SU8), also called hinge protein, is the only supernumerary subunit positioned on the top of MT-CYB in the IMS. This helical protein (3 helices) forms a hairpin-like structure due to the presence of two disulfide...
linkages. The involvement of this acidic protein (24 acidic residues) in the IMS CYC/CYC1 complex formation has been supposed, but remains unclear (Kim et al., 1989; Stonehuerner et al., 1985). The bc1 maturation and its catalytic activity are impacted by the loss of Qcr6p in yeast cells (Yang & Trumpower, 1994).

UQCRQ/Qcr8p (SU7), UQCR10/Qcr9p (SU10) and UQCR11/Qcr10p (SU11) are small proteins (less than 15 kDa) anchored to the IM by their single transmembrane domains and located at the periphery of the complex. UQCRQ/Qcr8p crosses the IM. Its C-terminal part forms a bent transmembrane helix exposed in the IMS side whereas its N-terminal end is located at the matrix side and serves as IM anchor for matrix subunits (Iwata et al., 1998; Xia et al., 1997). Additionally, UQCRQ/Qcr8p has been identified as a CoQ-binding protein (Usui et al., 1990). UQCR10/Qcr9p (SU10) is called CYC1-associated protein due to the interaction of its C-terminus with CYC1 in the IMS (Iwata et al., 1998). In yeast, Qcr9p is required for the formation of the dimeric bc1 complex and its deletion leads to an attenuated bc1 activity (Phillips et al., 1990). UQCR11/ Qcr10p (SU11) has one transmembrane helix. Although this subunit is required for the stable insertion of UQCRFS1 in the complex, it is not essential for the catalytic activity of the enzyme (Iwata et al., 1998; Brandt et al., 1994) . The delipidation of bovine bc1 complex and the preparation of yeast protein for crystal structure analysis induced the loss of the UQCR11 and Qcr10p, respectively, suggesting that these subunits are loosely associated to the bc1 complex (Schagger et al., 1990; Brandt et al., 1994; Islam et al., 1997).

### III.3. Assembly of bc1 complex

Yeast cells are able to survive in fermentable medium despite an impaired respiratory chain, therefore most of the work on the bc1 complex assembly has been performed in Saccharomyces cerevisiae. By using several yeast mutants with single or double bc1 complex subunit deletions and two-dimensional blue native/SDS-PAGE (2D BN/SDS-PAGE) analysis, a model describing a multi-step process involving several subcomplexes and assembly factors has been developed in the last years (Zara et al., 2007, 2004, 2009a; Conte et al., 2015; Gruschke et al., 2011; Hildenbeutel et al., 2014; Gruschke et al., 2012).
III.3.1. Early and late core subcomplexes

In yeast the assembly starts with the synthesis and membrane insertion of the mitochondrial-encoded subunit, cytochrome b (Cobp). Cobp can be associated with Qcr7p and Qcr8p to form the bc1 early core complex (Zara et al., 2007, 2004). In parallel, it was first proposed that Cyc1p could form a subcomplex with Qcr6p and Qcr9p and that the two core proteins Cor1p and Cor2p could interact with each other to generate a third subcomplex (Zara et al., 2004; Grivell, 1989; Fernández-Vizarra et al., 2009; Conte & Zara, 2011). However, recent data suggest that the core proteins Cor1p and Cor2p form a subcomplex with Cyc1p which is subsequently added to the bc1 early core complex to generate the late core bc1 complex or 500 kDa bc1 sub-complex (Zara et al., 2009b; Conte et al., 2015). Biochemical analysis revealed the presence of Qcr6p in this late core complex even if this protein is not required for its formation and stabilization (Conte et al., 2015; Zara et al., 2009b).

III.3.2. Rieske protein (UQCRFS1/Rip1p) insertion into late core subcomplex

The deletion of Qcr9p, Qcr10p and the catalytic subunit, Rieske protein (Rip1p), did not impair the formation of the late core complex, suggesting their incorporation in further steps of bc1 assembly (Zara et al., 2009b, 2009a; Conte et al., 2015). It was found that Qcr9p is added before Rip1p and that the incorporation of this catalytic subunit was required for the integration of Qcr10p into the mature and functional complex (Zara et al., 2009b). However, this last subunit is apparently devoid of any role in the respiratory activity and seems more involved in the stabilization of Rip1p in the complex (Iwata et al., 1998; Brandt et al., 1994). In addition, under conditions where the bc1 complex is severely impaired (deletion of core proteins or Cyc1p), a subcomplex including Qcr9p and Rip1p was identified (Conte et al., 2015; Zara et al., 2009a). The existence of this subcomplex in vivo has not been yet observed and confirmed. Nevertheless, in the bovine crystal structure, the transmembrane α-helix of the Rieske protein (UQCRSF1) interacts with the transmembrane helices of UQCR10/Qcr9p (Iwata et al., 1998). In addition, the deletion of Qcr9p in yeast impedes the proper insertion of the iron-sulfur cluster on Rip1p leading to a defective bc1 complex (Phillips et al., 1993; Zara et al., 2009b). To note, incorporation of the iron-sulfur cluster in Rip1p seems to occur after membrane insertion of the catalytic subunit to
stabilize it (Nett & Trumpower, 1999; Graham & Trumpower, 1991; Atkinson et al, 2011; Cruciat et al, 1999).

III.3.3. Dimerization of the bc1 complex

Crystal structures of the bc1 complexes from different organisms revealed a homodimeric organization of the bc1 complex (Iwata et al, 1998; Lange & Hunte, 2002; Xia et al, 1997; Zhang et al, 1998), however it is unclear when this dimerization occurs. The late core complex observed in yeast has a molecular mass of around 500 kDa, which can correspond to a direct dimeric bc1 core or to a monomer bc1 core with additional proteins (assembly factors or proteins of the other respiratory complexes). In this last option, the dimerization would occur after and may involve the Rieske protein due to its structure and disposition in the bc1 complex. Indeed, in crystal structures of the yeast and bovine bc1 complexes, the Rieske protein of each monomer are intertwined; its catalytic domain is located in one monomer and its transmembrane helix in the adjacent monomer (Iwata et al, 1998; Lange & Hunte, 2002; Xia et al, 1997; Zhang et al, 1998). However, the integration of the Rip1p in the late core complex induces a shift in the molecular mass of the complex which is too small to reflect a dimerization (Zara et al, 2009b). Recent data agree with a dimerization of bc1 complex during the early steps of the assembly. In yeast cells expressing both endogenous Cor2p and exogenous TAP tagged Cor2p, the Cor2p-TAP protein could be immunoprecipitated with the endogenous Cor2p (Conte et al, 2015). This result was similar when authors used a strain of yeast lacking Rip1p (Conte et al, 2015). This data suggests that the late bc1 core complex of 500 kDa possesses a dimeric structure and that Rip1p is not required for the dimerization. Thus, the molecular mechanism allowing the dimerization is still unknown.

Figure 10 represents the model proposed for human bc1 complex assembly by analogy with the yeast model.
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Figure 10: Bc1 complex assembly model. This model has been created by (Fernández-Vizarra & Zeviani, 2015) based on data obtained in yeast (Zara et al, 2007, 2009b; Atkinson et al, 2011; Gruschke et al, 2011, 2012; Wagener et al, 2011; Cui et al, 2012; Smith et al, 2012; Hildenbeutel et al, 2014). To start, the newly synthesized MT-CYB forms the early core complex with UQCRB and UQCRQ. This subcomplex can incorporate additional accessory subunits, UQCRC1, UQCRC2, and CYC1 which form another subcomplex. The late core complex incorporates UQCRH and seems to be already dimeric. However, the dimerization step is still unknown. The sequential incorporation of UQCR10, Rieske protein (UQCRFS1) and finally UQCR11 leads to the mature bc1 complex.

III.4. Nuclear-encoded accessory proteins involved in bc1 assembly

In addition to the eleven structural subunits, other proteins, called assembly factors, are involved in bc1 formation and/or stability. Whereas thirteen bc1 complex assembly factors were identified in yeast, only five were characterized in mammals: BCS1L (Fernandez-Vizarra et al, 2007; Hinson et al, 2007; Gil-Borlado et al, 2009), TTC19 (Ghezzi et al, 2011), UQCC1 and UQCC2 (Tucker et al, 2013) and LYRM7 (Sánchez et al, 2013).

III.4.1. UQCC1 and UQCC2

The bc1 complex assembly starts by the synthesis and membrane insertion of the sole mitochondrial encoded subunit, MT-CYB. The yeast Cobp assembly has been deeply studied. It includes a series of four subcomplex intermediates and requires three assembly factors: Cbp3p, Cbp6p and Cbp4p. Cbp3p and Cbp6p form a complex bound to the mitochondrial ribosome exit tunnel for efficient translation of the Cobp-encoding mRNA (Figure 11) (Gruschke et al, 2011, 2012). The Cbp3p–Cbp6p complex directly interacts with the newly synthetized and unhemylated Cobp. The generated intermediate, called 0, is released from the mitoribosome and stabilizes Cobp until its double hemylation and interaction with Qcr7p (Hildenbeutel et al, 2014). The
translation of the Cobp-encoding mRNA is stopped in the absence of Cbp3p-Cbp6p bound to the mitoribosome, in this way the sequestration of Cbp3p–Cbp6p in a Cobp–containing complex regulates Cobp synthesis (Gruschke et al., 2012). The second intermediate, called I, is obtained by incorporation of heme b₅ and the recruitment of Cbp4p which is required for the formation of a stable semi-hemylated Cobp (Hildenbeutel et al., 2014; Gruschke et al., 2011). Hemylation of the b₅ site triggers the release of Cbp3p–Cbp6p and the fully hemylated Cobp is stabilized by interaction with Cbp4p, Qcr7p and Qcr8p forming the intermediate II (Hildenbeutel et al., 2014; Gruschke et al., 2012). Cbp4p is released by the binding of Cor1p and Cor2p to the early core complex Cobp-Qcr7p-Qcr8p (Figure 11) (Gruschke et al., 2012).

Figure 11: Model for the assembly of yeast Cobp into early core complex. Cobp translated by mitoribosomes directly interacts with the Cbp3–Cbp6 complex, leading to intermediate 0 which stabilizes newly synthetized Cobp. The incorporation of the heme b₅ and of Cbp4p leads to the formation of intermediate I. Cbp4p maintains the semi-hemylated Cobp until its second hemylation at the b₅ site which induces the release of Cbp3p and Cbp6p. Cbp4p with Qcr7p and Qcr8p (intermediate II) stabilize the fully hemylated Cobp. The incorporation of additional subunits, Cor1p and Cor2p triggers the release of Cbp4p. Adapted from (Hildenbeutel et al., 2014).

Mammalian homologs have been identified for both Cbp3p and Cbp6p: namely UQCC1 and UQCC2 (Tucker et al., 2013).

UQCC2 is a matrix protein found tightly associated with the IM, it has been first found associated with the peripheral region of nucleoids (Cambier et al., 2012; Sumitani et al., 2009). However, a mutation on UQCC2 gene leading to an altered splicing of the transcript and the absence of the protein has been associated to a bc1 complex deficiency but not with a disturbance of mitochondrial nucleoids or mtDNA copy number (Tucker et al., 2013). Mitochondria of UQCC2-deficient patient display a low level of UQCRFS1 and of the dimeric bc1, both rescued by the overexpression of wild-type UQCC2 protein (Tucker et al., 2013).

UQCC1 is involved in bc1 assembly, as its yeast homolog Cbp3p. Its downregulation in mammalian cells induces an impaired bc1 activity associated with a slight reduction of bc1 mature complexes and an accumulation of low molecular weight UQCRC1-containing
intermediates (Tucker et al., 2013). As their yeast counterparts Cbp3p and Cbp6p, UQCC1 and UQCC2 interact together and are required for MT-CYB protein expression (Tucker et al., 2013; Gruschke et al., 2011). Indeed, UQCC1 has been shown to interact with nascent MT-CYB and UQCC2-deficient patient cells present a defect in MT-CYB protein level as observed in Cbp6-depleted yeast cells (Tucker et al., 2013; Gruschke et al., 2011). In addition, similarly to yeast proteins which are not found in the mature bc1 complex, UQCC1 and UQCC2 did not interact with UQCRC1, UQCRC2 and UQCRFS1 and UQCC1- and UQCC2-containing complexes were detected at lower molecular weights than the fully assembled bc1 complex (Hildenbeutel et al., 2014; Fernández-Vizarra & Zeviani, 2015). All together these data confirmed that UQCC1 and UQCC2 are assembly factors involved in the first step of the bc1 complex assembly (Figure 12).

The identification of the human functional equivalent of Cbp4p will be discussed in more details in the discussion section.

**III.4.2. BCS1L**

BCS1-Like (BCS1L) is the human orthologue of the yeast protein Bcs1p, with which it shares 50% of sequence identity (Petruzzella et al., 1998). BCS1L is a IM protein exposing its ATPase domain into the matrix (Petruzzella et al., 1998; Visapää et al., 2002). This protein is a part of the AAA (ATPases associated with a variety of cellular activities) family. AAA proteins assemble into oligomers to obtain usually a hexametric ring-configuration with a central cavity or pore and exhibit diverse functions including protein extraction from membranes for their processing and maturation (Truscott et al., 2010). Human and yeast Bcs1 proteins have been found in high molecular mass complexes suggesting an oligomeric configuration like most other AAA-proteins (Nouet et al., 2009; Hinson et al., 2007; Fernandez-Vizarra et al., 2007; Fernández-Vizarra et al., 2009). BCS1L is composed of distinct domains: a N-terminal domain involved in the targeting into the IM, a transmembrane helix, a Bcs1-specific domain with unknown function, and an AAA domain at C-terminus to bind and hydrolyze ATP (ATPase activity). This last domain is the most conserved amongst the different Bcs1 proteins, in contrast to the Bcs1-specific region where most of the variability occurs (Nouet et al., 2009; Cruciat et al., 1999; Fölsch et al., 1996). The yeast homolog Bcs1p has been more characterized than the human protein. Bcs1p-deficient yeast cells had no detectable bc1 activity, and presented an accumulation of the bc1 late core
intermediate and of the Rip1p in the matrix (Wagener et al., 2011; Cruciat et al., 1999; Zara et al., 2009b). In addition, Qcr9p and Bcs1p are both required for the integration of the catalytic subunit to the IM (Zara et al., 2009b). It has been proposed that Bcs1p forms a ring-like structure to mediate Rip1p translocation across the IM from the matrix (Wagener et al., 2011; Cruciat et al., 1999; Zara et al., 2009b). Bcs1p interacts in an ATP-dependent manner with Rip1p having a C-terminal domain folded (Wagener et al., 2011). Recently, it was shown that Bcs1p also interacts with the bc1 late core complex and with the mature dimeric bc1 (Conte et al., 2015). The group of Neupert proposed that after synthesis in the cytosol, Rip1p is imported in the matrix and acquires a suitable folding to be recognized by Bcs1p oligomer (Wagener et al., 2011). Then, once the Bcs1p/Rip1p complex interacts with the bc1 complex, Rip1p can be released and inserted into the respiratory complex. It was suggested that this release is driven by ATP hydrolysis and occurs when the hydrophobic segment of Rip1p reaches the transmembrane part of Bcs1p (Cruciat et al., 1999; Wagener et al., 2011). Human BCS1L is able to partially restore respiration of yeast lacking Bcs1p suggesting an overlapping function between the human and yeast proteins (de Lonlay et al., 2001; Visapää et al., 2002; Hinson et al., 2007). Beside functional data obtained by analogy with the yeast protein, studies of human BCS1L-linked diseases confirmed findings about its role on bc1 assembly. Numerous missense mutations (more than 20) have been reported in the BCS1L gene and lead to various biochemical impairments such as bc1 complex activity defect, increased production of ROS and iron overload (Ghezzi et al., 2011; Wagener & Neupert, 2012; Fernández-Vizarra & Zeviani, 2015). Most of BCS1L mutations are associated with a CIII deficiency, a reduced amount of UQCRFS1 and an accumulation of unstable bc1 late intermediate (de Lonlay et al., 2001; Visapää et al., 2002; Hinson et al., 2007; Fernandez-Vizarra et al., 2007; Kotarsky et al., 2010). Taken together, these observations suggest that, in humans as in yeast, BCS1L mediates the incorporation of the Rieske protein (UQCRFS1/Rip1p) in the bc1 complex. In the matrix compartment, a second UQCRFS1 chaperone has been identified: LYRM7/MZM1L (Figure 12) (Sánchez et al., 2013).

### III.4.3. LYRM7/MZM1L

LYR motif-containing protein 7 (LYRM7), or MZM1-Like (MZM1L), is the human homolog of the yeast protein Mitochondrial-Zinc-Maintenance 1 (Mzm1p) (Sánchez et al., 2013). Mzm1p has been characterized as a chaperone involved in bc1 assembly (Atkinson et al., 2010, 2011).
Mzm1p-deficient yeast cells had an impaired respiration with a specific defect in the bc1 complex. The loss of Mzm1p induced an accumulation of the bc1 late core intermediate and an attenuated level of Rip1p which seems prone to proteolytic degradation or temperature-induced aggregation (Atkinson et al, 2011, 2010). All this data suggest a role of Mzm1 in the late stage of bc1 assembly. It was shown that Mzm1p transiently interacts with the C-terminal globular domain of Rip1p and that this interaction stabilizes the catalytic protein before its membrane insertion by Bcs1p (Atkinson et al, 2011, 2010; Cui et al, 2012). LYRM7/MZM1L is a small soluble matrix mitochondrial protein which binds UQCRFS1 (Sánchez et al, 2013; Atkinson et al, 2010). Overexpression of LYRM7/MZM1L in HeLa cells induced an accumulation of UQCRFS1 in the matrix and prevented the insertion of the catalytic subunit in the IM. However, a slight LYRM7/MZM1L overexpression rescues UQCRFS1 membrane incorporation. In excess LYRM7/MZM1L seems to sequester UQCRFS1 in the matrix, indicating that the right stoichiometry of this assembly factor is required for its function (Sánchez et al, 2013). The loss of LYRM7 in HeLa cells causes a specific decrease of the total level of UQCRFS1 and a drastic reduction of bc1 mature complex associated to an impaired bc1 activity under heat stress, which is consistent with results obtained in yeast (Atkinson et al, 2011; Fernández-Vizarra & Zeviani, 2015). All together these data support that LYRM7/MZM1L acts as matrix chaperone of UQCRFS1 prior its membrane incorporation by BCS1L. In addition, an interaction between LYRM7/MZM1L and HSC20, a DnaJ type III co-chaperone implied in Fe-S cluster biogenesis, has been shown and suggests a potential role of LYRM7/MZM1L in Fe-S cluster delivery at UQCRFS1 (Maio et al, 2014). LYRM7/MZM1L belongs to the Leucine-Tyrosine-Arginine motif (LYRM) proteins which are exclusively found in eukaryotes and characterized by a N-terminal Lyr sequence followed by several basic arginine/lysine residues and an invariant phenylalanine (Angerer, 2013, 2015). The human genome contains at least 11 proteins of the LYRM superfamily which were predominantly identified as mitochondrial proteins (Angerer, 2015). Some of them are involved in iron-sulfur containing respiratory chain complexes, either as accessory subunits for the CI (NDUFB9/LYRM3 or NDUFA6/LYRM6) or as assembly factor, for the CII (SDHAF1/LYRM8 which also interact with HSC20) (Ghezzi et al, 2009; Angerer et al, 2011, 2014; Maio et al, 2014). The group of Winge has found that the tyrosine residue at the position 11 in the conserved Lyr motif of yeast Mzm1p is required for its function (Atkinson et al, 2010). The importance of the conserved Lyr motif in the mammalian protein has not yet been studied.
However, Invernizzi and colleagues identified the first *LYRM7/MZM1L* pathogenic mutation (c.73G>A) which induces a change of the aspartic residue in position 25 (p.Asp25Asn). The Asp25 residue is highly conserved. In Mzm1p-depleted yeast cells, the yeast D25N-Mzm1p mutant protein did not rescue bc1 activity and Rip1p stabilization, in contrast to the wild-type protein (Invernizzi et al., 2013; Sánchez et al., 2013). These results provided evidence that the Asp25 residue is required for *LYRM7/MZM1L* function on UQCRFS1. In summary, *LYRM7/MZM1L* functions within the matrix as a UQCRFS1 chaperone prior to its translocation by BCS1L (Figure 12).

### III.4.4. TTC19

TTC19 is a protein with a tetratricopeptide repeat (TPR) motif which is conserved among metazoa but not in other eukaryotes (plants and fungi) or prokaryotes. It is a 35 kDa IM protein which has been proposed to be involved in bc1 assembly (Ghezzi et al., 2011). It has been shown that mouse TTC19 interacts with UQRC1 and UQCRFS1 and a 2D BN/SDS-PAGE analysis indicates a potential interaction of TTC19 with the dimeric bc1 complex (Ghezzi et al., 2011). Most of reported TTC19 mutations in human are nonsense resulting in almost complete absence of TTC19 protein and leading to a bc1 complex deficiency (Melchionda et al., 2014; Ghezzi et al., 2011; Morino et al., 2014; Kunii et al., 2015; Koch et al., 2015; Nogueira et al., 2013; Mordaunt et al., 2015). TTC19-deficient cells obtained from fibroblasts or muscle of patients display a reduction of bc1 mature complex, more or less pronounced in the different patients and a clear accumulation of UQCR1-containing intermediates devoid of UQCRFS1 (Ghezzi et al., 2011; Melchionda et al., 2014). These observations suggest a role of TTC19 in an early step of bc1 assembly. The TPR motifs are regions of 34 residues length identified in organisms as diverse as bacteria and humans and are involved in a variety of functions including protein-protein interactions (Lamb et al., 1995). Some TPR domain-containing proteins were shown to act as co-chaperones, in concert with chaperones HSP70 and HSP90, on steroid receptor complexes. The TPR domains in these co-chaperones mediate the binding to HSP proteins and are involved in functional maturation of steroid receptors (Smith, 2004). Accordingly, the presence of TRP motifs in TTC19 could support its potential role as a mitochondrial chaperone involved in bc1 assembly (Ghezzi et al., 2011).
Further investigations are needed to understand the molecular mechanism involving TTC19 (Figure 12).

![Diagram](image)

**Figure 12: Global bc1 complex assembly model with assembly factors.** UQCC1 and UQCC2 form a complex which stabilizes the nascent and unhemylated MT-CYB at the tunnel exit of the mitoribosome. The heme b\(_1\) can be incorporated to MT-CYB. In yeast, the stabilization of the semi-hemylated Cobp requires Cbp4p. The identification of the human analogue of Cbp4p (represented by the question mark) will be addressed in the discussion section. The second hemylation of MT-CYB with heme b\(_2\) leads to the release of the UQCC1 and UQCC2. The fully hemylated MT-CYB can interact with UQCRB and UQCRQ. In yeast, the incorporation of Cor1p and Cor2p triggers the release of Cbp4p. The chaperone LYM7/MZM1L seems to stabilize the folded C-terminal domain of UQCRFS1 in the matrix prior its IM translocation by BCS1L which forms a hexameric oligomer. UQCRFS1 is incorporated in the bc1 complex after its ATP-dependent release from the BCS1L complex. The insertion of the pre-sequence of UQCRFS1 in the bc1 complex needs further investigations. TTC19 has been identified as a bc1 complex assembly factor required at the early stages of the assembly, however its molecular mechanism is currently unknown. This model was adapted from the model created by (Fernández-Vizarra & Zeviani, 2015) thanks to data obtained mostly in yeast and for some proteins in Human (Zara et al, 2007, 2009b; Atkinson et al, 2011; Gruschke et al, 2011, 2012; Wagener et al, 2011; Cui et al, 2012; Smith et al, 2012; Hildenbeutel et al, 2014).

### III.5. Role of lipids in bc1 complex stability and assembly

The bc1 complex is embedded in the IM and therefore interacts with phospholipids of this membrane. The total phospholipid content of solubilized bovine bc1 complex has been evaluated at 20–40 mol phospholipid/mol of CYC1 (Schagger et al, 1990) with 8–9 molecules of tightly
bound CL (Hayer-Hartl et al., 1992) and around 100-200 molecules of more loosely associated PE and PC (Musatov & Robinson, 1994). Crystal structure of yeast bc1 complex highlighted up to 13 tightly bound phospholipid molecules mostly identified in the matrix leaflet of the IM (Lange et al., 2001; Solmaz & Hunte, 2008; Palsdottir & Hunte, 2004). Lange and colleagues identified in yeast bc1 complex a PI molecule wrapped around the transmembrane helix of Rip1p and interacting with Cyc1p (Lange et al., 2001). Alteration by site-directed mutagenesis of the primary ligands of this PI (lysine residues on Cyc1p) destabilizes the interaction of Rip1p subunit with the bc1 complex indicating the structural role of this phospholipid. Authors suggested that this interhelical PI molecule could stabilize the transmembrane region of Rip1p during the movement of the flexible extrinsic domain of the catalytic protein (cf section II.2.1) (Lange et al., 2001).

Data obtained following site-directed mutagenesis on several phospholipid-binding sites in the yeast bc1 complex indicates that these phospholipids are important for the structural and functional integrity of the enzyme (Lange et al., 2001; Palsdottir & Hunte, 2004). The same applies to phospholipid interactions in the bovine bc1 complex, because phospholipid removal leads to the destabilization and inactivation of the enzyme (Yu & Yu, 1980; Gomez & Robinson, 1999; Fry & Green, 1981; Schagger et al., 1990). The bovine bc1 activity has been rescued by phospholipids mixtures necessarily including CL or its precursor PG supporting the critical functional role for CL bound to bovine bc1 (Gomez & Robinson, 1999). The first CL site has been identified on the crystal structure of the yeast enzyme, close to the Qi-site at the entrance site for protons (Lange et al., 2001). This CL interacts with Cyc1p, Qcr7p and Cobp and appears to have two roles in the bc1 complex: a structural one at the Qi-site and a functional one in proton uptake (Lange et al., 2001; Klingen et al., 2007). The second CL site has also been found thanks to X-ray structure of yeast bc1 complex, in the cavity at dimer interface and its head group is stabilized via interactions with residues of Cor1p and Cobp from the same monomer and by additional nonpolar interactions with other subunits like Cyc1p (Palsdottir & Hunte, 2004). Acyl chains of this CL seem flexible into the lipid bilayer and could promote substrate diffusion from the membrane to the active site (Palsdottir & Hunte, 2004). Mutations of lysine residues involved in interaction of CL with Cyc1p cause a reduced amount of Cobp and Cyc1p and destabilize the bc1 complex indicating the structural role of this CL (Palsdottir & Hunte, 2004). To identify new CL-binding
sites, Arnarez and coll. used coarse-grain molecular dynamics simulations (CGMD), a technique often used to study dynamics, structure, and stability of membrane–protein systems (Figure 13).

![Image of simulation box of the bovine bc1 complex used by Arnarez and colleagues to identify the CL binding sites by coarse-grain molecular dynamics simulations. The dimeric bc1 complex is represented in green, in a membrane bilayer where 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (POPC) are in gray/white and CLs in red/orange. The aqueous phase (water molecules and sodium ions) is shown in blue. Some of the aqueous phase is removed to ease the view of the system.](image)

Arnarez and coll. found six CL binding sites per monomer (CLI-CLVI), including the two sites already identified in yeast, in both yeast and bovine bc1 complexes (Arnarez et al, 2013b). Although CLs are present at both matrix and IMS sides of CIII, all the CL binding sites identified by simulation are situated on the matrix side. The table 2 summarizes the localization of each site identified in bc1 complex, the number of CL which can be hosted by each site and the bc1 subunits interacting with the CL. These binding sites and their localization are conserved across species suggesting an important role in the bc1 complex. Their localization at the matrix side could support their involvement in proton uptake (Arnarez et al, 2013b; Lange et al, 2001)
Table 2: CL-binding sites identified by X-ray structures analysis and coarse-grain molecular dynamics simulations in the bovine bc1 complex (Arnarez et al., 2013b; Lange et al., 2001; Palsdottir & Hunte, 2004)

III.6. Bc1 complex deficiencies

Among mitochondrial diseases, isolated bc1 complex deficiencies are very rare OXPHOS disorders diagnosed in comparison with isolated CI deficiencies (Janssen et al., 2006; Diaz et al., 2011). Currently, mutations in eight genes encoding for bc1 catalytic and structural subunits as well as for its assembly factors have been reported in human as associated with mitochondrial diseases.

Around 40 disease-associated mutations, including frameshift, termination mutations, deletions, and missense mutations, have been identified in the $MT$-$CYTB$ gene (OMIM 516020 and reviewed by (Meunier et al., 2013)). Mutations in this catalytic subunit lead to a broad spectrum of clinical phenotypes: exercise intolerance, lactic acidosis, mitochondrial myopathy, Leber’s hereditary optic neuropathy (LHON), encephalopathy, cardiomyopathy and severe neonatal polyvisceral failure (Andreu et al., 1999; Johns & Neufeld, 1991; Blakely et al., 2005; Keightley et al., 2000; Fragaki et al., 2009; Meunier et al., 2013).
Mutations have been also reported in nuclear genes encoding bc1 subunits: the supernumerary subunits UQCRB (OMIM 191330) (Haut et al., 2003) and UQCRQ (OMIM 612080) (Barel et al., 2008), the core subunit UQCRC2 (OMIM 191329) (Miyake et al., 2013) and the catalytic subunit CYC1 (OMIM 123980) (Gaignard et al., 2013). A patient with a 4-bp deletion in UQCRB gene resulting in an abnormal elongation at the C-terminal part of the protein cumulated with a modification of seven aa developed hypoglycemia and lactic acidosis (Haut et al., 2003). A missense mutation in UQCRQ gene has been detected in patients suffering from severe psychomotor retardation, and dementia. The serine residue at position 45 was replaced by a phenylalanine impacting significantly the 3D structure of UQCRQ and potentially its suitable insertion in the bc1 complex (Barel et al., 2008). In 2013, Miyake and colleagues identified a pathological mutation in UQCRC2 which induces a tryptophan to arginine change at position 183 of the protein (Miyake et al., 2013). Similar clinical phenotypes were observed in patients with UQCRB and UQCRC2 mutations; hypoglycemia, lactic acidosis, and ketosis (Miyake et al., 2013; Haut et al., 2003). Hyperammonemia and frequent metabolic crises were also observed in patients with UQCRC2 defects (Miyake et al., 2013). Different missense mutations in CYC1 have been identified in two unrelated patients which presented similar clinical features: ketoacidotic and lactic acidotic encephalopathy and insulin-responsive hyperglycemia (Gaignard et al., 2013). Fibroblasts of both patients showed a severe reduction in CYC1 and an impaired bc1 complex assembly. Overexpression of wild-type CYC1 protein in patient fibroblasts rescued the bc1 defect and confirmed that the responsibility of CYC1 mutations in bc1 complex deficiency (Gaignard et al., 2013).

As mentioned in section II.4, mutations have been also identified in bc1 assembly factors, and biochemical analyses of patient’s mitochondria provided important information to complete the bc1 assembly model. More than 25 pathological mutations were identified in the three different domains of BCS1L (Fernández-Vizarra & Zeviani, 2015). A clinical heterogeneity was observed between patients with BCS1L mutations. Indeed, BCS1L mutations have been associated with neonatal tubulopathy, encephalopathy and liver failure (OMIM 606104) (de Lonlay et al., 2001), or with isolated encephalopathy (Fernandez-Vizarra et al., 2007). A missense mutation resulting in a S78G aa change in BCS1L had been identified in Finnish patients which have a severe multivisceral disorder called GRACILE syndrome (growth retardation,
aminoaciduria, cholestasis, iron overload, lactic acidosis and early death MIM 603358) (Visapää et al, 2002). The Björnstad syndrome (OMIM 262000), characterized by sensorineural hearing loss and pili torti, has been also associated to two mutations in BCS1L (Hinson et al, 2007; Fellman, 2002). Depending on the mutation, BCS1L-mutated fibroblasts show mitochondrial defects which can be linked to: an impaired import and assembly of the BCS1L, a defective incorporation of the UQCRFS1 in the bc1 complex leading to a CIII deficiency or an increase of ROS production associated to a respiratory chain destabilization (Fernandez-Vizarra et al, 2007; Hinson et al, 2007; Morán et al, 2010; Kotarsky et al, 2010). To note, tissue specific effects of different BCS1L mutations have been reported on bc1 complex assembly. Indeed, fibroblasts of GRACILE patients have no abnormality in bc1 complex structure and function, while in mitochondria-enriched organs such as liver, kidney and heart, a low level of BCS1L and UQCRFS1 as well as fully bc1 complex assembled were observed (Kotarsky et al, 2010; Hinson et al, 2007). Additionally, liver iron overload is a characteristic of GRACILE patients, but not Björnstad syndrome patients, suggesting another cellular function of BCS1L in iron metabolism. Since 2011, several mutations in TTC19 have been reported, leading to premature protein truncation or nonsense-mediated RNA decay (OMIM 613814) (Ghezzi et al, 2011; Nogueira et al, 2013; Morino et al, 2014; Kunii et al, 2015; Atwal, 2014). These pathological mutations cause heterogeneous clinical phenotypes including progressive cerebellar ataxia and dysarthria, mental impairment, psychiatric manifestations, progressive neurodegenerative disorder, and Leigh syndrome (Ghezzi et al, 2011; Nogueira et al, 2013; Morino et al, 2014; Kunii et al, 2015; Atwal, 2014). Most patients with TTC19 mutations show a bc1 complex deficiency, as expected due to the function of TTC19 as bc1 assembly factor (Ghezzi et al, 2011; Nogueira et al, 2013; Morino et al, 2014; Kunii et al, 2015; Atwal, 2014). As previously mentioned, a mutation has been reported on LYRM7/MZM1L inducing an aa change at the level of a highly conserved residue and causing a severe, early onset encephalopathy with lactic acidosis (OMIM 615831) (Invernizzi et al, 2013). Tucker and colleagues reported a bc1 deficiency associated-mutation on UQCC2 in a patient suffering of severe intra-uterine growth retardation, neonatal lactic acidosis and renal tubular dysfunction (OMIM 614461) (Tucker et al, 2013). The point mutation caused a splicing defect and an absence of detectable UQCC2 protein in fibroblasts of the patient (Tucker et al, 2013).
IV. Respiratory chain supercomplexes (RCS)

The organization of the respiratory complexes in the IM is the subject of numerous studies. In 1986, the group of Gupte purified the five respiratory complexes as active individual entities and proposed then the “fluid model” (Figure 14 A) (Hackenbrock et al, 1986). In this model, the electron transfer is supported by random and transient collisions between the independent respiratory complexes diffusing freely in the IM and the two mobile carriers, CYC and CoQ (Hackenbrock et al, 1986). However the development of BN gel electrophoresis gave evidences of interaction between respiratory complexes in yeast and bovine mitochondria, suggesting a “solid model” for the OXPHOS organization (Figure 14 B) (Schägger & Pfeiffer, 2000; Schägger, 2001). Since then, supramolecular assemblies, called respiratory chain supercomplexes (RCS) or respirasomes have also been identified in diverse organisms such as bacteria (Stroh et al, 2004), plants (Eubel et al, 2003), and human (Schägger et al, 2004). In 2008, the group of Enríquez proposed a model of OXPHOS organization called “plasticity model” which combines the two pre-existing models. In this model, respiratory complexes are found both as single complexes and as RCS, which allow adaptation of the OXPHOS to changing conditions (Figure 14 C) (Acín-Pérez et al, 2008).

Figure 14: Schematic representation of the three proposed models for the OXPHOS complexes organization
A) The “fluid” model: each respiratory complex acts as an active individual entity and the electron transfer is supported by random collisions between the complexes and the mobile carriers, CYC and CoQ (Hackenbrock et al, 1986). B) The “solid” model: complexes I, III and IV interact to form a RSC called respirasome. In this context, the
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Electron transport occurs within the RSC between complexes and carriers leading to substrate channeling (Schägger & Pfeiffer, 2000; Schägger, 2001). The "plasticity" model which combines both previous models. Individual complexes co-exist with RCS in the IM, allowing a dynamic formation of RCS. The electron transport occurs within RCS by substrate channeling and between individual complexes thanks to different pools of CYC or CoQ (Acín-Pérez et al., 2008). Respiratory complexes are colored in blue (CI), red (CII), yellow (CIII, the bc1 complex), purple (CIV) and green (CV, ATP synthase). Coenzyme Q (CoQ) is represented by small red dots and cytochrome c (CYC) as blue-filled triangles. Adapted from (Dudkina et al., 2008).

EM studies on mammalian mitochondria supported biochemistry data and confirmed the presence of large RCS containing a monomer of CI, a dimer of the bc1 complex and a monomer of CIV (Schäfer et al., 2006; Althoff et al., 2011). This I/III2/IV RCS corresponds to the major CI-containing RCS observed in mammals, however other RCS have also been observed (Schägger, 2001). Indeed, CI is hardly detected as free complex (only 14-16% of CI), because it is mostly found in RCS containing the bc1 complex and zero to four copies of CIV: I/III2 (17% of CI) and I/III2/IV1-4 (around 70% of CI) (Acín-Pérez et al., 2008; Moreno-Lastres et al., 2012; Schagger & Pfeiffer, 2001; Schägger, 2001). Additionally, fifty percent of bc1 complex has been identified in large RSC involving CI, whereas forty percent was detected in free dimer and only ten percent associated to a monomer of CIV (III2/IV) (Moreno-Lastres et al., 2012). CIV is predominantly found as free enzyme (65-70%) or as CI-containing RSC (I/III2/IV1-4) (30%). Only a few copies are detected bound to the bc1 complex (III2/IV) (1-3% of CIV) (Moreno-Lastres et al., 2012). To note, the presence of CII in these RCS is still under debate. With the exception of Acín-Pérez and colleagues, most groups failed to identify CII in mammalian RCS in normal physiological conditions (Acín-Pérez et al., 2008; Muster et al., 2010; Schagger & Pfeiffer, 2001; Moreno-Lastres et al., 2012). In the case of Saccharomyces cerevisiae which lacks CI, the bc1 complex forms RSC with one or two copies of CIV, III2/IV and III2/IV2 (Heinemeyer et al., 2007).

IV.1. Are RCS respiratory active entities?

In-gel and spectrophotometric activity assays in mammals confirmed that each complex involved in both isolated I/III2 and I/III2/IV RCS was active (Schäfer et al., 2006). However the NADH:ubiquinol reductase activity of CI and the cytochrome c reductase activity of bc1 complex were two-fold more active in RSC indicating the importance of such structures for the respiratory complex activity (Schäfer et al., 2006). Importantly, the isolated I/III2/IV RCS was able to carry out respiration in the presence of CYC, CoQ and NADH, whereas a mix of the three isolated complexes in same conditions was unable to respire (Acín-Pérez et al., 2008). These results
indicated that the association of respiratory complexes in supramolecular organization is required for OXPHOS respiratory function (Acín-Pérez et al., 2008). Acín-Pérez and colleagues also confirmed that the I/III$_2$/IV RCS were functional units of respiration, also called respirasomes, by detecting CYC and CoQ in isolated RCS (Acín-Pérez et al., 2008).

**IV.2. Functional roles of RCS**

**IV.2.1. Kinetic advantage: channeling**

The functional role of RCS is widely questioned in literature and remains unclear. One of the proposed roles of RCS is to improve the catalytic activity of complexes by enhancing the electron flow via substrate channeling. Substrate channeling is the direct transfer of an intermediate between the active sites of two enzymes without its release into the bulk phase (Spivey & Ovádi, 1999). The 3D structure of I/III$_2$/IV RCS indicated distances of 13 nm between the ubiquinol-binding sites of CI and the bc1 complex and 10 nm between the CYC-binding sites of CIV and the bc1 complex (Figure 15) (Dudkina et al., 2011; Althoff et al., 2011). These proximities minimize the distance travelled by electrons from their entry site in the matrix arm of CI to their final site in CIV and provide structural evidences for substrate channeling (Althoff et al., 2011; Schäfer et al., 2007). Moreover, the presence of CYC and CoQ in purified mammalian I/III$_2$/IV RCS supports the substrate channeling idea (Acín-Pérez et al., 2008; Althoff et al., 2011). Although the existence of the ubiquinone channeling between CI and the bc1 complex is now assumed, the confirmation of substrate channeling between the bc1 complex and CIV is still under investigation (Bianchi et al., 2004; Genova & Lenaz, 2014).
IV.2.2. OXPHOS adaptation to metabolic needs

The ratio of NADH:FAD electrons feeding the ETC changes according to the nature of the available substrate. It is higher when the respiratory substrate is glucose compared to fatty-acids. In absence of glucose, Lapuente-Brun and coll. observed a reduced level of CI-containing RSC associated to an increase of free bc1 complex (Lapuente-Brun et al, 2013). They demonstrated that these changes in the OXPHOS organization provide a mechanism to ensure efficient use of available substrate. Indeed, bc1 complexes can only receive electrons from the oxidation of NADH when they are bound to CI whereas they can receive only electrons provided by oxidation of FADH₂ when they are free or bound to CIV (Figure 16) (Lapuente-Brun et al, 2013). Therefore, under starvation, the composition of RSC is changed to provide more free bc1 complex accessible to electrons coming from FADH₂ (Lapuente-Brun et al, 2013). In this way, the dynamic RCS formation and assembly would have a role in the cell adaption to substrate availability (Lapuente-Brun et al, 2013).
IV.2.3. Reactive oxygen species limitation

It was postulated that the improvement of the electron flow by channeling in RCS could limit ROS formation (Schägger, 2001; Lenaz & Genova, 2012; Seelert et al, 2009). Maranzana and coll. confirmed that disruption of the CI-containing RCS by dodecyl-β-D-maltoside (DDM) treatment enhance the generation of ROS from CI (Maranzana et al, 2013). Further investigations are needed to confirm this functional role.

IV.2.4. Structural advantage: protein stability and assembly scaffold

The role of RSC in the stabilization of individual complexes has often been proposed, especially for CI. Numerous interactions observed between both bc1 complex and CIV with CI, in the 3D structures of RCS, suggested that these supercomplexes keep CI in a stable conformation (Schäfer et al, 2006). Beside this structural observation, biochemistry studies on mammalian cells
lacking fully assembled bc1 complex or CIV revealed that both of these complexes were required for the maintenance of CI (Acín-Pérez et al., 2004; Diaz et al., 2006; Li et al., 2007; D’Aurelio et al., 2006). These data were in accordance with combined CI and bc1 complex defects observed in the most of patients with mutations in CIII-specific genes (Bruno et al., 2003; Lamantea et al., 2002; Morán et al., 2010; Fernandez-Vizarra et al., 2007; Tucker et al., 2013; Schägger et al., 2004). The majority of patients with mutations in CI-specific genes had isolated CI deficiencies indicating that the assembly of CI was not required for the assembly and stability of the bc1 complex and CIV (Schägger et al., 2004; Fassone & Rahman, 2012). However, some studies revealed a combined CI and CIII deficiency, in patients with mutations on NDUFS4 gene encoding for a subunit of CI (Budde et al., 2000, 2003; van den Heuvel et al., 1998) and recently, a secondary CIV deficiency was observed in a patient with a mutated CI chaperone (Saada et al., 2012). Two possible explanations are proposed for these rare multiple OXPHOS deficiencies in CI-mutated patients: mutations could affect subunits of CI involved in direct interactions with other complexes within the respirasome or a dysfunctional CI cause oxidative damage leading to instability of other OXPHOS complexes (Genova & Lenaz, 2014; Fassone & Rahman, 2012).

IV.3. Assembly of RSC

Several groups work to understand the mechanism of RSC assembly and two pathways have been proposed. In 2009, Enríquez and colleagues proposed that the formation of RCS would be subsequent to the assembly of the three individual complexes (Figure 17 A) (Acín-Pérez et al., 2008). This model was based on pulse-chase experiments where the 13 mtDNA-encoded proteins were labelled and their time course incorporation into respiratory complexes and RCS was monitored by 2D BN/SDS-PAGE electrophoresis. By this approach, they observed that individual complexes were labeled several hours before their incorporation into RCS (Acín-Pérez et al., 2008). However, this model was confronted to recent data obtained by the group of Ugalde which monitored the formation of newly synthetized OXPHOS complexes and RCS in cell lines where respiratory complexes are earlier depleted thanks to doxycycline treatment (Figure 17 B) (Moreno-Lastres et al., 2012). According to their results, they proposed the following multi-step model for RCS assembly: (1) CI is first assembled as an inactive intermediate lacking a NADH dehydrogenase catalytic module; (2) In parallel, isolated complexes III and IV are assembled until reaching a threshold that probably triggers the accumulation of free subunits and
assembly intermediates from these two complexes; (3) The CI-intermediate is then used as scaffold for the incorporation of these bc1 complex and CIV free subunits and assembly intermediates; (4) When these complexes are fully assembled on the CI-intermediate, the NADH dehydrogenase catalytic core can be added to finalize the CI assembly as well as the functional RCS assembly (Moreno-Lastres et al., 2012). This model could explain the structural dependence of CI on the presence of assembled bc1 and CIV complexes and give an additional function of RCS as a platform for the sequential assembly of functional CI (Moreno-Lastres et al., 2012). Additionally, the presence of individual fully assembled bc1 complex and CIV explained why the human CI deficiencies rarely lead to combined OXPHOS defects (Shoubridge, 2012).

Figure 17: Models proposed for respirasome assembly and the assembly factors involved. A) Model proposed by the group of Enríquez which suggested that the complexes are first assembled individually before the formation of RSC (Acín-Pérez et al., 2008). The bc1 complex (CIII) and CIV could interact directly with CI to generate the respirasome (I/III2/IV) or they could form an III2/IV RSC before their association with CI. B) Model proposed by the Uglade’s group where an inactive CI-intermediate lacking the NADH dehydrogenase core serves as scaffold for the incorporation of the bc1 complex and CIV subunits (Moreno-Lastres et al., 2012). In this model, the bc1 complex and CIV are first fully assembled until reaching a threshold which leads to the accumulation of free subunits and/or assembly intermediates of these complexes. These complex fractions are then added to the CI-intermediate. When the bc1 complex and CIV are fully assembled in the CI-arm, the NADH dehydrogenase core can be incorporated to complete CI assembly and respirasome formation. SCAF1 and HIG1A or HIG2A have been identified as potential assembly factors involved in the interaction of the bc1 complex with CIV. HIG1A or HIG2A seem also required for CIV assembly. The molecular mechanisms used by these RSC assembly factors are still unknown. Adapted from (Moreno-Lastres et al., 2012; Porras & Bai, 2015).
IV.4. Assembly factors and stabilizing factor

As explained before, numerous assembly factors have been found for OXPHOS complexes. The identification of similar factors involved in RCS assembly has recently been the focus of most studies on RSC. Two schools of thought co-exist concerning factors which can be considered as RCS chaperones. Either, the same set of assembly factors is common to individual complexes and RSC, or a set exclusive to RSC.

IV.4.1. Cardiolipin

In the literature, CL appears to play a central role in RCS formation and stabilization. The first evidences were obtained in yeast, where a destabilization of III₂/IV₂ RCS was observed in CL-lacking yeast mutants (Zhang et al., 2002; Pfeiffer et al., 2003) and in yeast cells with mutations at the specific CL-binding sites of the bc₁ complex (Wenz et al., 2009). More recently Bazan and colleagues reported the first in vitro CL-dependent reconstitution of the functional III₂/IV₂ RCS from individual bc₁ complex and CIV in proteoliposomes and then highlighted the essential role of CL in the formation of yeast RSC (Bazán et al., 2013). The requirement of CL in the stabilization of the human RSC is evidenced by the analysis of Barth syndrome patients' mitochondria which displayed lower CL content. They presented a modification in CL acyl chain composition associated to a decrease in I/III₂/IV RCS and an increase of free CIV (McKenzie et al., 2006; Schlame & Ren, 2006). Additionally, structural studies of the purified mammalian respirasome revealed gaps between transmembrane domains at the interface of individual complexes which may be filled with lipids. It is estimated that around 200 CL molecules are present in these structures (Althoff et al., 2011; Dudkina et al., 2011). It was hypothesized that the presence of CL within RCS could provide flexible interactions between the respiratory complexes which would facilitate the dynamic formation and dissociation of RSC in response to environmental conditions (Mileykovskaya & Dowhan, 2014). To visualize how CL could be involved in interaction between the bc₁ complex and CIV, Arnarez and colleagues performed coarse-grained-molecular dynamic simulations with both complexes. Their data systematically show CL inside the formed RSC and some of these CL seems to be shared by both complexes (Figure 18). Therefore, CLs could be glue proteins of the bc₁ complex and CIV together stabilizing the III₂/IV RSC. (Arnarez et al., 2013b).
IV.4.2. Rcf1p/Rcf2p (HIGD1A, HIGD2A)

Recently three groups identified two yeast proteins, called Rcf1p and Rcf2p or respiratory supercomplex factors 1 and 2, potentially involved in interaction between the bc1 complex and CIV (Strogolova et al., 2012; Chen et al., 2012; Vukotic et al., 2012). Both proteins are members of the hypoxia inducible genes (Hig-1) family. Rcf1p is an integral protein of 18 kDa with two predicted transmembrane helices and C- and N-termini exposed in the IMS (Strogolova et al., 2012; Chen et al., 2012; Vukotic et al., 2012). Similarly, Rcf2p consists of two predicted transmembrane helices and has a size of 25 kDa (Strogolova et al., 2012; Vukotic et al., 2012). Rcf1p and Rcf2p have been copurified with yeast RCS (Strogolova et al., 2012; Vukotic et al., 2012) and appeared to interact independently with both bc1 complex and CIV (Strogolova et al., 2012; Chen et al., 2012; Vukotic et al., 2012). While Rcf2p is present in RCS, its role in these supramolecular complexes is still unknown as its loss did not induce change in their assembly (Vukotic et al., 2012; Strogolova et al., 2012). Experiments of Rcf1p depletion and overexpression suggest its role in RCS formation and maintenance. Indeed, the three groups observed a severe reduction of the level of III2/IV2 in Rcf1p depleted yeast cells (Strogolova et al., 2012; Vukotic et al., 2012; Chen et al., 2012), and the Rutter group obtained a subtle increase of III2/IV2 RSC by overexpression Rcf1p in wild-type yeast (Chen et al., 2012). The loss of Rcf1p also induced a
specific decrease in CIV activity, which suggests an additional role of Rcf1p in CIV assembly (Strogolova et al., 2012; Chen et al., 2012). Consistent with this notion, Vukotic and colleague proposed that Rcf1p is a structural subunit of CIV required for the assembly of the Cox13p subunit (Vukotic et al., 2012). Interestingly, they also reported an heterogeneity in CIV composition in the IM because they identified a form of CIV lacking Cox13p which is unable to be incorporated in RSC (Vukotic et al., 2012). The regulation of Cox13p incorporation into CIV could be one of the ways used by Rcf1p to mediate RSC formation. Rcf1p could also mediate interaction between the bc1 complex and CIV by acting as a bridge. Indeed, Rcf1p is able to interact independently with the bc1 complex and with CIV, and it was speculated that Rcf1p could be localized at the interface of these two complexes (Strogolova et al., 2012; Chen et al., 2012). Two mammalian orthologues have been identified for Rcf1p: HIGD1A (RCF1a) and HIGD2A (RCF1b). Both orthologues were identified as IM proteins displaying a topology similar as the yeast protein (Vukotic et al., 2012). Analysis of in vitro HIGD1A and HIGD2A mitochondrial import indicates that both proteins may be part of human CIV (Vukotic et al., 2012). However HIGD1A cannot complement the growth phenotype of Rcf1p-depleted yeast, whereas HIG2A was able to partially rescue the growth defect (Vukotic et al., 2012). In addition only the depletion of HIGD2A caused a decrease of mammalian CIV-containing RCS, but without affecting the amount of free CIV (Chen et al., 2012). These preliminary data suggest that the mammalian protein could be important for CIV-containing RCS maintenance, but further investigations are needed to understand the molecular mechanism (Figure 17).

IV.4.3. COX7A2L/SCAF1

Using mouse models, two independent studies identified a new RSC assembly factor: cytochrome c oxidase subunit VIIa polypeptide 2-like (COX7A2L), also named supercomplex assembly factor 1 (SCAF1) (Lapuente-Brun et al., 2013; Ikeda et al., 2013). They found that SCAF1 only interacts with CIV-containing RCS and is required for stable interaction between the bc1 complex and CIV without affecting the stability of the individual complexes (Lapuente-Brun et al., 2013; Ikeda et al., 2013). The Enríquez’ group identified a mutation in SCAF1 which leads to a short and unstable protein unable to trigger interactions between the bc1 complex and CIV. Scaf1-mutated mouse fibroblasts display mitochondria without CIV-containing RCS, whereas cells lines with wild-type SCAF1 have all RCS types. The overexpression of wild-type SCAF1 into
Scaf1-mutated mouse fibroblasts rescues the formation of CIV-containing RCS confirming the role of the wild-type SCAF1 (Lapuente-Brun et al, 2013). In agreement with these results, Ikeda and colleagues also observed a decrease of CIV-containing RCS in Scaf1-knockout mice and showed that the overexpression of SCAF1 in HEK293T cells stimulates the respirasome assembly (Figure 17) (Ikeda et al, 2013). By investigating the respiration rates of Scaf1-mutated and non-mutated cells, the Enríquez’ group discovered that the assembling of CIV into RCS thanks to SCAF1 defines three CIV populations: CIV associated with the respirasome I/III2/IV which can receive electrons exclusively from NADH; a fraction of CIV bound to the bc1 complex which used only electrons from FAD-dependent enzymes, and free CIV which are able to receive electrons from NADH and FADH2 (Figure 16) (Lapuente-Brun et al, 2013). Beside the identification of a new RSC chaperone, this study highlighted also the importance of RSC and their dynamic adaptation to an available substrate (Lapuente-Brun et al, 2013).
Aim of Thesis
As described in detail in the Introduction, mitochondria are involved in many cellular processes and more particularly in the energy metabolism by hosting the respiratory complexes. More than 80 different proteins, mostly encoded by the nuclear genome, are involved in respiratory complexes. Their assembly, which is complicated and not well understood, requires additional nuclear-encoded proteins called assembly factors (Ghezzi & Zeviani, 2012). Mutations in numerous genes encoding OXPHOS subunits or assembly factors have been reported in patients with mitochondrial disorders (Ghezzi & Zeviani, 2012; Femández-Vizarra & Zeviani, 2015). Additionally, it is now demonstrated that the respiratory complexes associate to form RSC in the IM (Schäfer et al., 2006; Althoff et al., 2011). The RSC biogenesis as well as the potential accessory proteins required for their formation are still the subjects of active investigations (Acín-Pérez et al., 2008; Lapuente-Brun et al., 2013; Moreno-Lastres et al., 2012). Therefore, we can speculate that several assembly or stabilizing factors of OXPHOS complexes and RSC are still unknown and that their identification and characterization represent major challenges to decipher molecular mechanisms which can be responsible of mitochondrial diseases.

Many efforts are being made to catalog all the mitochondrial proteins. It has been estimated that the mammalian mitochondrial proteome is composed of approximately 1,500 proteins (Taylor et al., 2003b, 2003a; Pagliarini et al., 2008; Meisinger et al., 2008). In 2008, Pagliarini and coll. identified 1,100 mitochondrial proteins which seems to correspond to 85% of the total mitochondrial proteome (Pagliarini et al., 2008). Among these proteins, around one-fifth lacks experimental validations (Calvo & Mootha, 2010; Pagliarini et al., 2008). Therefore, some work is still required to further characterize the currently identified proteins and to complete the mitoproteome with new mitochondrial proteins. The goal of the CALIPHO (Computational Analysis and Laboratory Investigation of Proteins of Human Origin) group is to select and characterize such proteins lacking experimental validation (Mary et al., 2012; Bontems et al., 2014; Salleron et al., 2014).

The aim of this thesis was to characterize C11orf83 which is a small protein of unknown function having a putative mitochondrial localization. Initially, this protein had been predicted by bioinformatic and large scale experimental studies as a secreted protein with a potential signal peptide of 23 aa (Clark et al., 2003; Zhang and Henzel, 2004). However, C11orf83 was later found in mitochondrial membrane fractions from HeLa S3 cells (Catherman et al., 2013).
Importantly, in a large scale mouse knock-out screen, depletion of C11orf83 was shown to be embryonic lethal (Tang et al., 2010), suggesting an essential cellular function.

Therefore, we first solved the discrepancy in terms of C11orf83 cellular localization. As we confirmed the mitochondrial localization of C11orf83, we then explored the mitochondrial phenotypes induced by the depletion of C11orf83 and investigated more precisely the biological function of C11orf83 at mitochondrial level.
I. **C11orf83 is an integral mitochondrial IM protein facing the IMS**

C11orf83 is a protein of 10.1 kDa (93 aa) which is conserved in vertebrates. As shown in the multiple sequence alignment in Figure 19, there is a similarity of 50 % between human and *Xenopus laevis* C11orf83. In addition, secondary structure prediction (PSIPRED) indicated the presence of conserved secondary structures (Figure 19). However, no obvious functional domain was found (InterProScan) and no similarity to any protein with a resolved 3D structure could be retrieved by HHpred.

![Sequence alignment of human C11orf83 with its orthologs.](image)

We first analyzed the gene expression profile of C11orf83 by PCR on panels of human tissue cDNA (Figure 20 A) and we observed that C11orf83 is widely expressed among the different tissues tested. After confirming the expression of C11orf83 in the HeLa cell line commonly used in our laboratory, we decided to use this cell line for the further characterization of C11orf83 (Figure 20 B).

![C11orf83 is widely expressed in human tissues](image)
Bioinformatic analysis, as well as a large scale experimental study, originally predicted that C11orf83 was a secreted protein due to the presence of a potential signal peptide of 23 aa in length (Figure 19) (Clark et al., 2003; Zhang & Henzel, 2004). Therefore, we studied the potential secretion of C11orf83. HeLa cells were transfected with a plasmid encoding this protein with a C-terminal V5 epitope tag (C11orf83-V5). The culture medium (supernatant) and the cellular lysate were harvested and analyzed by western blot with an anti-V5 antibody (Figure 21). As control, we used the C-terminal V5 tagged form of interleukin-6 (IL6), a secreted cytokine (Scheller et al., 2011). In addition, β-catenin detection by immunoblotting and Ponceau staining of the PVDF membrane were used as loading control for the cell lysates and supernatant samples, respectively. The large pink bands in the supernatant samples revealed by Ponceau staining correspond to the BSA contained in the cell culture medium. In these conditions, we detected IL6-V5 in the cell lysate and in the culture medium, confirming its secretion. In contrast, we did not observe C11orf83 in the supernatant but a clear signal in cellular lysate, suggesting that C11orf83 was expressed but not secreted (Figure 21).

Figure 21: Assay of C11orf83 secretion. HeLa cells were transfected with C11orf83-V5 or IL6-V5. After 48 h of transfection, the culture medium (supernatant) and cell lysates were harvested and analyzed by immunoblotting. C11orf83-V5 and IL6-V5 were detected using an antibody against their tag V5. B-catenin was used as a loading control for the cell lysates whereas a Ponceau staining was performed to control the loading of supernatant samples.

C11orf83 was recently identified among the membrane proteins in a proteomic study of crude mitochondria extracts from HeLa S3 cells (Catherman et al., 2013). We decided to analyze the localization of C11orf83 in HeLa cells by confocal microscopy. We identified C11orf83 in mitochondria because of a co-localization with COX4, a mitochondrial protein (Figure 22 A). Therefore, the localization of C11orf83 within mitochondria was determined by subcellular fractionation experiments. After Na₂CO₃ treatment of the mitochondria-enriched fraction, integral
membrane proteins, like TOMM20, were found in the pellet and soluble proteins, like DIABLO, were retained in the supernatant. C11orf83 was found in the membrane protein pellet (Figure 22 B), suggesting that it is a mitochondrial integral membrane protein, in agreement with the results of the aforementioned proteomic study (Catherman et al., 2013). To determine C11orf83 membrane topology, we performed a proteinase K protection assay. Mitochondria-enriched fractions from HeLa cells were treated with proteinase K, in the presence or absence of saponin which is used to permeabilize the OM. In contrast to the IM protein TIMM44, which is oriented towards the mitochondrial matrix, C11orf83 was digested by proteinase K in the presence of saponin, as it was the case for DIABLO, a soluble protein from the IMS, and OPA1, a mitochondrial IM protein facing the IMS ((Figure 22 C, lane 5).

Figure 22: **C11orf83 is a mitochondrial IM protein** A) Immunofluorescence analysis of C11orf83 localization in HeLa cells by confocal microscopy. Endogenous C11orf83 (anti-C11orf83 pAb, green) was co-localized with COX4 (anti-COX4 pAb, red). Merged color image is shown. (Scale bars: 10 µm in image, 2 µm in inserts). B) The mitochondria-enriched fraction from HeLa cells was subjected to Na$_2$CO$_3$ extraction and analyzed by immunoblotting. C11orf83 and TOMM20 were retained in the insoluble membrane pellet (P), whereas the soluble protein DIABLO was released into the supernatant (S). (I Input). C) Mitochondria-enriched fractions from HeLa cells were digested by proteinase K in the absence (lane 2) or presence of saponin (lane 5) and analyzed by immunoblotting. Controls were analyzed lanes 1, 3, 4 and 6. C11orf83 was only digested by proteinase K in the presence of saponin (lane 5), indicating that C11orf83 is in the IM and faces the IMS. (M matrix).
Taken together, these data indicate that C11orf83 is a mitochondrial IM protein facing the IMS. We performed the same assay using HeLa cells overexpressing the C-terminal V5 tagged form of C11orf83 (C11orf83-V5), and observed similar localization and membrane topology (Figure 23 A, B and C).

**Figure 23:** C-terminal V5-tagged C11orf83 has the same subcellular localization as endogenous C11orf83. A) Immunofluorescence analysis of V5-tagged C11orf83 localization in HeLa cells by confocal microscopy. C11orf83-V5 (anti-V5 mAb, green) was co-localized with COX4 (anti-COX4 mAb, red). Merged color image is shown. (Scale bars: 10 µm in image, 2 µm in inserts). B) The mitochondria-enriched fraction from HeLa cells transfected with C11orf83-V5 was incubated with 0.1 M Na₂CO₃ (pH 11), centrifuged and analyzed by immunoblotting. C11orf83-V5 and COX4 were retained in the insoluble membrane pellet (P), whereas the soluble protein DIABLO was released into the supernatant (S). (Input) C) Mitochondria-enriched fractions from HeLa cells transfected with C11orf83-V5 were digested by proteinase K in the absence (lane 2) or presence of saponin (lane 5) and analyzed by immunoblotting. Controls were analyzed lane 1, 3, 4 and 6. C11orf83-V5 was digested by proteinase K only in presence of saponin, indicating that it localizes in the IM and faces the IMS. (M Matrix)

Therefore, we hypothesized that the hydrophobic N-terminal part of C11orf83, predicted to be a secretory signal peptide (Clark et al., 2003; Zhang & Henzel, 2004), would rather serve as a mitochondrial targeting signal and a membrane-anchoring region. To test this hypothesis, we expressed a modified form of GFP with this peptide fused at its N-terminal (WT-N23-GFP) in HeLa cells. By confocal microscopy, we observed a clear co-localization of the mitochondrial COX4 signal with the WT-N23-GFP signal (Figure 24 A and B). The mitochondria-enriched fraction from HeLa cells expressing WT-N23-GFP was then subjected to Na₂CO₃ treatment. WT-N23-GFP was found in the pellet corresponding to the membrane protein fraction (Figure 24 C). These data suggest that this N-terminal sequence is sufficient to target and anchor C11orf83 to the mitochondrial IM. At the exception of Danio rario, we identified a conserved arginine-lysine (RK) motif in this N-terminal region (Figure 19, red box). We substituted these 2 residues either...
by alanine or by aspartic acid (Figure 24 A), and analyzed the localization of these mutated N23-GFP forms in HeLa cells. Mutations in the RK motif abrogated N23-GFP targeting to the mitochondria and induced a cytoplasmic localization (Figure 24 D). We observed similar results with RK-mutated C11orf83-V5 (Figure 24 E), which confirmed the key role of these two positively charged aa for the correct mitochondrial localization of C11orf83.

**Figure 24: C11orf83 is targeted and anchored in the mitochondria IM by its N-terminal hydrophobic sequence.** A) The NH2-terminal part of C11orf83 (23 aa) was fused to GFP for expression in HeLa cells (WT-N23-GFP). Introduced mutations are shown in red in the corresponding sequences. B) Immunofluorescence analysis of WT-N23-GFP localization in HeLa cells by confocal microscopy. WT-N23-GFP fusion protein was colocalized with COX4 (anti-COX4 pAb, red). Merged color image is shown. C) The mitochondria-enriched fraction from HeLa cells transfected with WT-N23-GFP was subjected to Na2CO3 extraction and analyzed by immunoblotting. WT-N23-GFP (labelled with anti-GFP) and COX4 were retained in the insoluble membrane pellet (P). The minor band detected by the anti-GFP antibody may reflect protein degradation. D) Immunofluorescence analysis of HeLa cells transfected with WT-N23-GFP or its mutated forms by confocal microscopy. The WT-N23-GFP fusion protein was colocalized with COX4 (anti-COX4 pAb, red), whereas R5AK6A- and R5DK6D-N23-GFP fusion proteins were mislocalized in the cytosol. Merged color images are shown. E) Immunofluorescence analysis of HeLa cells transfected with WT or mutated C11orf83-V5 plasmids by confocal microscopy. V5-tagged proteins are stained in green (mAb V5 tag) and COX4 in red. While WT-C11orf83-V5 showed a mitochondrial colocalization with COX4, the R5AK6A- and R5DK6D-C11orf83-V5 proteins displayed a cytosolic staining. Merged color images are shown. (Scale bars: 10 µm in images, 2 µm in inserts).
II. C11orf83 downregulation impairs mitochondrial functions

To investigate the biological function of C11orf83 at the cellular level, we engineered HeLa cell lines by stably downregulating C11orf83 using shRNA technology. Two cell lines, called sh-1 and sh-2, were chosen due to their absence of detectable C11orf83 protein expression (Figure 25 A). We noticed that C11orf83 downregulation was more stable in sh-2 cells than in sh-1 cells (Figure 25 B). On freshly thawed cell lines (less than six passages), we observed similar growth curves for cells downregulating C11orf83 and controls (Figure 25 C). However, we noticed that after six passages, C11orf83-deficient cells showed a significantly reduced cell growth compared to controls (Figure 25 D). These data suggest that the C11orf83 loss has a potential impact on resistance to in vitro cellular aging.

Figure 25: Establishment of C11orf83-deficient cell lines. A) Western blot analysis of HeLa cells downregulating C11orf83 (sh-1 and sh-2) compared to HeLa cells expressing a control shRNA (sh CTL) and to control HeLa cells without shRNA (HeLa WT). β-catenin was used as a loading control. B) Two western blot analyses of C11orf83-downregulated and control cell lines after more than 6 passages of cultures. A weak level of C11orf83 is detectable in sh-1 compared to sh-2 (black arrows), suggesting that the sh-2 cell line is more stable than the sh-1 cell lines. GAPDH was used as a loading control. C) Growth curve of HeLa WT, sh-1, sh-2 and sh CTL cells at early passages after thawing (mean ± SD (n=3)). D) Growth curves of HeLa WT, sh-1, sh-2 and sh CTL cells at late passages after thawing (> 6) (mean ± SD (n=3)).

Since C11orf83 was shown to be mitochondrial and several studies proposed a connection between aging and mitochondria dysfunctions (Bratic & Larsson, 2013; Shigenaga et
al, 1994; Yen et al, 1989; Short et al, 2005; Stocco et al, 1977), a deeper analysis of the impact of C11orf83 depletion on mitochondrial physiology has been performed. To comply with the American Type Culture Collection (ATCC)'s instructions (American Type Culture Collection, 2003) which recommend the use of cell lines within five passages to ensure reliable and reproducible results, all the following experiments were performed with freshly thawed cells. As mitochondria play a key role in apoptosis and ATP production, we first monitored the sensitivity of these cell lines to apoptosis, and their ATP levels. Apoptosis was induced by actinomycin D, and monitored by flow cytometry using annexin V labelling. The assay specificity was confirmed by the inhibition of annexin V-staining in the presence of z-VAD-FMK, a pan-caspase inhibitor. A significant increase in annexin V positive cells was observed in C11orf83-deficient cells (Figure 26 A). These results showed that the knock-down of C11orf83 induced a higher sensitivity to this apoptotic stimulus. Next, we measured the ATP levels of each cell line using a luciferase reporter assay. The C11orf83-deficient cell lines sh-1 and sh-2 showed a 40% and 30% reduction in ATP levels compared to controls, respectively (Figure 26 B). Since the cellular ATP is mainly provided by mitochondria via the ETC, we wondered if the observed decrease in cellular ATP could be due to an impaired respiration. Using a cell-based assay, we showed that the loss of C11orf83 induced a significant decrease of basal respiration (Figure 26 C). Taken together, these results suggest that C11orf83 is important for proper mitochondrial physiology.
Figure 26: Mitochondrial functions of C11orf83-deficient cell lines. A) Analysis of actinomycin D (Actino D)-induced apoptosis sensitivity of HeLa WT, sh-1, sh-2 and sh CTL cells. Cells were treated with Actino D or methanol (control) in the absence or presence of the caspase inhibitor z-VAD-FMK. Cell death was quantitated by flow cytometry using an annexin V-conjugated APC staining kit (mean ± SD (n=3)). B) Cellular ATP levels of HeLa WT, sh-1, sh-2 and sh CTL cells (mean ± SD (n=3)). Before the ATP assay, cells were cultured in a medium without glucose in the presence of oligomycin (to confirm the specificity of the assay by inhibiting ATP synthesis) or DMSO (as control). C) Analysis of the basal respiration of HeLa WT, sh-1, sh-2 and sh CTL cells. Results are expressed as the mean ± SD of percentages of the basal respiration of sh CTL cells (n=3).

III. C11orf83 depletion induces defects in RSC

As the loss of C11orf83 induced a decrease in basal respiration, we investigated its potential effects on individual respiratory complexes. In Table 3, we summarize the activities of the five respiratory complexes in knock-down and control cells measured by spectrophotometry analysis. All complexes demonstrated a significantly lower activity in C11orf83 knock-down cells than in controls.
Results

<table>
<thead>
<tr>
<th></th>
<th>HeLa WT</th>
<th>sh-1</th>
<th>sh-2</th>
<th>sh CTL</th>
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<tbody>
<tr>
<td>Complex I</td>
<td>26.4 ± 1.6</td>
<td>20.1 ± 1.0 *</td>
<td>17.2 ± 2.9 **</td>
<td>31.3 ± 1.9</td>
</tr>
<tr>
<td>Complex II</td>
<td>7.8 ± 3.5</td>
<td>4.8 ± 1.5 *</td>
<td>4.0 ± 1.0 **</td>
<td>8.7 ± 1.8</td>
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<tr>
<td>Complex III</td>
<td>94.0 ± 13.9</td>
<td>62.5 ± 23.4 *</td>
<td>70.9 ± 23.7 *</td>
<td>128.2 ± 46.0</td>
</tr>
<tr>
<td>Complex IV</td>
<td>100.1 ± 13.0</td>
<td>61.2 ± 8.8 *</td>
<td>79.9 ± 3.5 *</td>
<td>121.7 ± 21.3</td>
</tr>
<tr>
<td>Complex V</td>
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<td>13.4 ± 5.6 *</td>
<td>10.6 ± 0.3 *</td>
<td>28.3 ± 10.4</td>
</tr>
</tbody>
</table>

Values are normalized to citrate synthase activities and reported as mean ± SD. (n=3)

* P < 0.05, ** P< 0.01 compared to sh control values

Table 3: Enzymatic activities of mitochondrial respiratory complexes in WT, sh control and C11orf83-deficient cells (sh-1, sh-2)

Then, we examined the effects of C11orf83 downregulation on the steady state levels of proteins that form ETC complexes. Immunoblotting on whole cell lysates, under denaturing conditions, showed that the loss of C11orf83 did not affect the amount of representative subunits for each complex (NDUFV2 for CI, SDHB for CII, UQCRC1 for CIII, COX4 for CIV and ATP5B for CV) (Figure 27 A).

To determine whether the assembly of ETC complexes and RSC was affected in C11orf83-depleted cells, we performed BN gel electrophoresis followed by immunoblotting on isolated mitochondria solubilized by DDM. In this condition, CII and CV were extracted as individual complexes (visualized with antibodies against SDHB for CII and ATP5B for CV) (Figure 27 B). Complexes I and IV and the complex III dimer were also extracted as individual complexes, and additionally found as III2/IV, I/III2 and I/III2/IV RSC (visualized with antibodies against NDUFB6 for CI, UQCRC1 for CIII, COX4 for CIV) (Figure 27 C). C11orf83 depletion caused no apparent change in the detectable amounts of Complexes I, II, III2, IV and V and of I/III2 RSC (Figure 27 B, C). In contrast, we observed a significant reduction in the amounts of I/III2/IV and III2/IV RSC, with a more pronounced effect for the III2/IV RSC (Figure 27 C arrows D and E).
Figure 27: C11orf83 depletion induces defects in the RSC. A) Western blot analysis of the steady-state levels of representative subunits of respiratory complexes (NDUFV2 for Complex I (CI), SDHB for Complex II (CII), UQCRC1 for the Complex III (CIII), COX4 for Complex IV (CIV) and ATP5B for Complex V (CV)) in HeLa WT, sh-1, sh-2 and sh CTL cells. β-catenin was used as a loading control. B, C) DDM-solubilized isolated mitochondria from HeLa WT, sh-1, sh-2 and sh CTL cells were analyzed by BN-PAGE and immunoblotted using antibodies against respiratory complexes. Differences between cell lines are shown by arrows. D) Quantification of the I/III:IV RSC using antibody against NDUFB6 (mean ± SD (n=3)). E) Quantification of the III:IV RSC using antibody against UQCRC1 (mean ± SD (n=3)).
IV. C11orf83 interacts with the bc1 complex and is involved in the early stages of bc1 assembly

To understand the cause of the reduced amounts of I/III_2/IV and III_2/IV RSC, we solubilized mitochondria using digitonin, which better preserves RSC integrity than DDM, and performed 2D BN/SDS-PAGE followed by immunoblotting. When this protocol was applied to control cells, CI (visualized with antibody against NDUFV2) was found uniquely in the I/III_2/IV RSC, whereas CIV and CIII (visualized with antibodies against COX4 and UQCRRC1, respectively) were found both as individual complexes and in III_2/IV and I/III_2/IV RSC, as expected from the literature (Schagger & Pfeiffer, 2001; Moreno-Lastres et al, 2012) (Figure 28 A). In mitochondria from sh-2 cells solubilized with digitonin, UQCRRC1 was predominantly found in the I/III_2/IV RSC, and we observed a significant reduction in the amounts of CIII and III_2/IV RSC (Figure 28 A arrows, B, C). This observation suggests that CI sequesters the limited amount of assembled CIII/bc1 complex and III_2/IV RSC available in sh-2 cells, similarly to what was reported for other assembly defects of the bc1 complex (Lapuente-Brun et al, 2013) or CIV (Lazarou et al, 2009). The fact that we could observe free CIII/bc1 complex as well as III_2/IV RSC in DDM-solubilized mitochondria from C11orf83-depleted cells (Figure 27 C) was probably due to the dissociation of the I/III_2/IV RSC in these extraction conditions. Taken together, our data indicates that the loss of C11orf83 induces a deficiency in CIII/bc1 complex assembly.
**Figure 28: C11orf83 is involved in bc1 complex assembly**

A) Digitonin-solubilized isolated mitochondria from sh CTL and sh-2 cells were analyzed by 2D BN/SDS-PAGE. The bc1 complex was detected with antibodies against UQRC1, UQCRB and CYC1. Antibodies against NDUF2, SDHB and COX4 were used to detect Complexes I, II and IV, respectively. SDHB was used as a loading control since CII assembly was shown to be unaffected by C11orf83 depletion and C11orf83 as a control for downregulation (Figure 27 B). B) Quantification of the bc1 complex (III2) and C) III2/IV were performed using antibody against UQRC1. D) Quantification of CYC1-containing complex intermediates at low molecular weights was performed using antibody against CYC1 (D). (Mean ± SD (n=3))

In the yeast model, it was first proposed that the Qcr7p/Qcr8p/Cobp subcomplex, called the bc1 core complex, interacts sequentially with two other preformed subcomplexes, Cyc1p/Qcr6p/Qcr9p and Cor1p/Qcr2p, to form intermediate complexes (Zara et al., 2004; Grivell, 1989; Fernández-Vizarra et al., 2009; Conte & Zara, 2011). However, new evidences suggested that Cyc1p could be associated with the core proteins Cor1p and Cor2p. This new subcomplex could be interacting with the bc1 core complex with a subsequent incorporation of individual subunit Qcr6p and Qcr9p (Zara et al., 2009b; Conte et al., 2015). The late stages of the bc1 complex assembly correspond to the incorporation of Rip1p and Qcr10p (Smith et al., 2012; Zara et al., 2007; Conte et al., 2015). By selecting UQCRC1 (human ortholog of Cor1p), UQCRB (human ortholog of Qcr7p) and CYC1 (human ortholog of Cyc1p), we can study the early stages of the bc1 complex assembly whatever the CYC1-containing subcomplexes proposed by both models. Using antibodies against UQCRB and CYC1, we observed a clear decrease in the bc1 complex and III2/IV RSC in C11orf83-depleted cells, which is in agreement with the results...
obtained with the anti-UQRC1 antibody (Figure 28 A). At lower molecular weights, we noticed an additional accumulation of complex intermediates containing CYC1 but not UQRC1 or UQCRB. The composition of this subcomplex is still unknown, it could represent an oligomer of unassembled CYC1, a subcomplex involving only CYC1 and UQRC2 or an equivalent to the Cyc1p/Qcr6p/Qcr9p subcomplex proposed in the first bc1 assembly model (Figure 28 A arrow, D). Importantly, overexpression of a C11orf83-V5 form (translated from a mRNA which is not targeted by the shRNA) in sh-2 cells clearly decreased the accumulation of CYC1-containing complex intermediates (Figure 29 A and B). This result confirms that C11orf83 is an assembly factor involved in the early stages of the bc1 complex assembly.

Figure 29: The exogenous expression of C11orf83-V5 rescues the CYC1-containing intermediates accumulation caused by the loss of C11orf83 A) Digitonin-solubilized isolated mitochondria from sh CTL cells, sh-2 cells and sh-2 cells transfected with C11orf83-V5 were analyzed by 2D BN/SDS-PAGE. The bc1 complex was detected with antibodies against UQRC1 and CYC1. The presence of endogenous C11orf83 or its V5-tagged form was detected by using an antibody against C11orf83. B) Quantification of the CYC1-containing complex intermediates at low molecular weights using antibody against CYC1 (mean ± SD (n=3)).

We next studied the effect of C11orf83 deletion on the steady-state levels of several bc1 complex subunits by performing immunoblotting on whole cell lysates under denaturing conditions. We observed a pronounced reduction in the UQCRB (human ortholog of Qcr7p, Figure 30 A, B) level and a slight reduction in the UQCRQ (human ortholog of Qcr8p, Figure 30 A, B).
A, C) level in C11orf83-depleted cells, suggesting a reduction in the amount of the bc1 core complex. The protein levels of all other analyzed subunits were not modified compared to controls (Figures 30 A). Therefore, C11orf83 seems to be specifically involved in the stabilization of the bc1 core complex at the early stage of the bc1 complex assembly. The accumulation of the CYC1-containing complex intermediates observed in the absence of C11orf83 (Figure 28 A, D) could be due to limiting amounts of the bc1 core complex.

Figure 30: C11orf83 is involved in the stabilization of the bc1 subunits UQCRQ and UQCRB. A) Western blot analysis of the amount of several bc1 complex subunits in whole lysates from HeLa WT, sh-1, sh-2 and sh CTL cells, under denaturing conditions. β-catenin was used as a loading control and C11orf83 as a control for downregulation. B) UQCRB and C) UQCRQ protein levels were quantified using antibodies against UQCRB and UQCRQ. (Mean ± SD (n=3))

As we showed that C11orf83 is involved in bc1 complex assembly, we wanted to check whether it interacts with this complex. A first indication came from the BN-PAGE and 2D-BN/SDS-PAGE analyses, where C11orf83 was detected in bands migrating at the same molecular weights than CIV, CIII\textsubscript{2} and III\textsubscript{2}/IV RSC (Figure 28 A and 31 A). An additional fourth band at around 400 kDa is more visible in the BN-PAGE analysis (Figure 31 A), which could be correspond to a dimer of CIV. These observations indicate that C11orf83 could interact with the bc1 complex and with CIV. To test this hypothesis, we conducted co-immunoprecipitation experiments. The bc1 complex and CIV were successfully immunoprecipitated from HeLa WT mitochondria-enriched fractions solubilized using DDM, as shown by western blot analysis using
antibodies against UQCRFS1 and COX4, respectively (Figure 31 B). C11orf83 was specifically co-purified with the bc1 complex, which indicates that C11orf83 interacts with this complex. In these conditions, we did not detect any interaction between C11orf83 and CIV (Figure 31 B).

**Figure 31:** C11orf83 is present in four high-molecular-weight complexes and interacts with the bc1 complex. A) DDM-solubilized isolated mitochondria from HeLa WT, sh-1, sh-2 and sh CTL cells were analyzed by BN-PAGE and immunoblotted using antibody against C11orf83. SDHB was used as control loading. B) Co-immunoprecipitation analyses. The bc1 complex and the CIV were immunoprecipitated from DDM-solubilized HeLa WT mitochondria-enriched fractions. Western blot analysis was performed after SDS-PAGE using anti-COX4 for Complex IV (CIV), anti-UQCRFS1 for the bc1 complex (CIII) and anti-C11orf83.

**V. C11orf83 is a CL-binding protein involved in cristae maintenance**

Phospholipids, and in particular CL, the mitochondria-specific phospholipid, are essential for the stabilization and function of the bc1 complex (Wenz *et al*., 2009) as well as for the stabilization of the III$_2$/IV$_2$ RSC in yeast (Bazán *et al*., 2013) and the III$_2$/IV and I/III$_2$/IV RSC in mammalian cells (Althoff *et al*., 2011; McKenzie *et al*., 2006). As we have observed a bc1 complex deficiency with an impaired formation of the III$_2$/IV RSC in C11orf83-deficient cells, we hypothesized that C11orf83 could either be involved in phospholipid metabolism or interact with mitochondrial phospholipids. Therefore, we studied the lipid composition of mitochondria-enriched fractions from either sh-2 or control cells by thin layer chromatography (TLC). Mitochondrial fractions from both cell lines displayed similar levels of CL, PE, PI and PC. They
had equivalent CL to PE ratios, when measuring either TLC spot intensity (sh CTL: 0.71±0.11, sh-2: 0.67±0.13) or phosphate content (sh CTL:0.23±0.03, sh-2: 0.15±0.05) (Figure 32).

Figure 32: TLC analysis of the lipid composition of mitochondria-enriched fractions from sh-2 or sh CTL cells.

Quantification of total CL in both cell lines by mass spectrometry (MS) confirmed this result (sh CTL: 1.4 ± 0.2 nmol/mg of protein, sh-2: 1.8 ± 0.4 nmol/mg of protein). CL has four acyl chains which can vary in length and unsaturation. Further analysis of mass spectra to evaluate CL acyl composition revealed that some CL species of the C64 (4xC16), C66 (3xC16,1xC18) and C68 (2xC16,2xC18) clusters were slightly but significantly increased in sh-2 cells (C64:4, C66:5, C66:6, C68:5, C68:6 and C68:7, Figure 33 A and B). However, there was no shift in unsaturation, only an increase in the abundance of the C16-enriched CL clusters. Our result indicates that the depletion of C11orf83 induces a shift in the CL composition of mitochondria to shorter acyl chains.
Figure 33: C11orf83 loss induces changes in fatty acid CL composition. A) Representative CL mass spectra from sh CTL and sh-2 cells. The different CL clusters are indicated using CXX (where the number “XX” designates the amount of carbon atoms in the fatty acid side chains). m/z 619.4 and m/z 665.4 are the CL (CL IS) and phosphatidylglycerol internal standards (PG IS), respectively. B) Quantification of CL species in sh-2 and sh CTL cells (mean ± SD (n=4)). CL species are represented as CXX:Y, where XX designates the total amount of carbon atoms and Y corresponds to the total number of double bonds in the fatty acid side chains. There is a subtle but significant increase in the abundance of the C64, C66 and C68 clusters.

To analyse the potential binding of C11orf83 to mitochondrial phospholipids, we monitored the direct interaction of recombinant GST-C11orf83 protein on phospholipid blots (Dowler et al., 2002). While recombinant GST did not produce any signal in this assay, GST-C11orf83 specifically bound to CL, PA and sulfatide (Figure 34 A). C11orf83 binding to sulfatide, a sphingolipid, is most probably not physiologically relevant as this lipid is not known to be
present in mitochondria, but found in the plasma membrane of most eukaryotic cells (Xiao et al., 2013). In contrast, C11orf83 may physiologically interact with CL and/or with PA, which is synthesized in the mitochondrial OM and transferred in the IM to be converted to CL (Chakraborty, 1999). To determine the CL-binding domain of C11orf83, we generated recombinant GST proteins corresponding to several subportions of C11orf83 and observed their potential interaction with phospholipids (Figure 34 B). GST-Δ23C11orf83, C11orf83 protein depleted of its N-terminal transmembrane domain, showed a similar binding to CL, PA and sulfatide than C11orf83 full-length (Figure 34 B and C), indicating that interaction with CL is not mediated by the C11orf83 membrane-anchoring domain. As most of CL-binding proteins interact with CL by their α-helices (Palsdottir & Hunte, 2004; Arnarez et al., 2013a; Liu et al., 2005; Lange et al., 2001), we analyzed the phospholipid-binding ability of predicted C11orf83 α-helices, either separately (GST-Helix 2 and GST-Helix 3), or together (GST-Helix 2-Helix 3) (Figure 34 B and C). Fusion proteins GST-Helix 2 and GST-Helix 3 did not produce any signal in this assay, in contrast to the protein GST-Helix 2-Helix 3 which displayed a similar binding to CL, PA and sulfatide than the GST-C11orf83 full length (Figure 34 C). Taken together, our experiments showed that the combination of helix 2 and helix 3 (aa 23-80) is necessary and sufficient for CL-binding (Figure 34 B) and could play a role in the CL-binding of the bc1 complex or/and in the III2/IV RSC stabilization.
C11orf83 is a CL-binding protein. A) Recombinant GST-C11orf83 was used to probe membrane lipid strips. Recombinant GST was used as control of non-specific binding. B) Schematic representation of the different GST fusion proteins of full length and truncated C11orf83, with aa numbering. C) Lipid binding assays of GST-Δ23C11orf83, GST-Helix 2, GST-Helix 3 and GST-Helix 2-Helix 3. (TG triglyceride, DAG diacylglycerol, PS phosphatidylserine, PG phosphatidylglycerol, PI(4)P phosphatidylinositol-4-phosphate, PI(4,5)P phosphatidylinositol-4,5-phosphate, PI(3,4,5)P phosphatidylinositol-3,4,5-phosphate)

Studies on the composition of the CL binding sites of the respiratory complexes highlighted that the side chains of positively charged aa (lysine and arginine) were frequently involved in electrostatic bonds with CL due to CL negative charge (Arnarez et al., 2013a; Pöyry et al., 2013; Arnarez et al., 2013b). Phenylalanine and leucine residues were also found to be involved in CL binding sites of complexes I and III (Arnarez et al., 2013a). In addition, aa which contribute to CL-binding are often well conserved over species (Arnarez et al., 2013b). According to these data, we introduced several mutations in GST-C11orf83 (L24A, K35A, K40A, R55A, R77A Figure 19, black arrows) and assessed the CL binding of these mutated forms. Unfortunately, none of these point mutations impaired the CL-binding ability of C11orf83 (Figure 35).
Results

Figure 35: Lipid binding assays of recombinant GST-C11orf83 proteins displaying individual mutation; L24A, K35A, K40A, R55A and R77A, compared to the wild type GST-C11orf83. GST was used as control of non-specific binding.

Recently, Cogliati and coll. showed that the cristae morphology is linked to the assembly and stability of RSC (Cogliati et al., 2013). Since we observed defects in bc1 complex–containing RSC in the absence of C11orf83, we suspected modifications in the IM morphology. Using EM, we confirmed a disorganization of the IM ultrastructure and cristae architecture in C11orf83-depleted cells compared to control cells (Figure 36).

Figure 36: C11orf83 is involved in cristae maintenance. Electron microscopy micrograph of thin sections of sh CTL (left quadrant) and sh-2 (right quadrant) cells showing the ultrastructure of mitochondria. Arrows indicate mitochondria. (Scale bars: 1 µm).

This observation is similar to what was reported with cells depleted for OPA1, a GTPase with the same localization and topology than C11orf83 (Olichon et al., 2002, 2003; Kushnareva et
In addition, C11orf83-deficient cells present several features which are similar to what is observed in OPA1-downregulating cells, including impaired respiration, decreased ETC enzymatic activities and high apoptosis sensitivity (Kushnareva et al., 2013; Olichon et al., 2003) (Figure 26 A and C, Table 3). Because OPA1 was shown to be involved in high molecular weight complexes (Cogliati et al., 2013), we analyzed whether C11orf83 depletion induced changes in mitochondrial OPA1-containing complexes. Figure 37 shows the migration pattern of OPA1 in C11orf83 depleted cells compared to sh control cells in 2D-BN/SDS-PAGE. Whereas OPA1 was found in complexes of the expected size of around 480 and 720 kDa in control cells (Cogliati et al., 2013), OPA1 was aberrantly localized in complexes of various molecular masses in C11orf83-depleted cells. As OPA1 is located in the cristae junction and seems to form a molecular bridge between the adjacent membranes of the cristae (Frezza et al., 2006), the formation of aberrant complexes containing OPA1 may be due to the disorganization of the IM ultrastructure observed in C11orf83 deficient cells (Figure 36).

Figure 37: Analysis of OPA1-containing complexes in sh CTL and sh-2 cells. Isolated mitochondria were solubilized by digitonin and analyzed by 2D BN/SDS-PAGE. Western blot analysis was performed using antibodies against C11orf83 to control the downregulation, SDHB for the loading control and OPA1.

**VI. C11orf83 is cleaved by OMA1 upon mitochondrial stress, like OPA1**

OPA1 is known to undergo proteolytic processing by several proteases, either constitutively or upon mitochondria depolarization (Griparic et al., 2007; Cipolat et al., 2006; Head et al., 2009; Ehses et al., 2009). For example, under stress conditions induced by carbonyl cyanide m-chlorophenyl hydrazone (CCCP), OPA1 is cleaved and inactivated by the zinc metalloprotease OMA1 which consequently promotes mitochondrial fragmentation (Ehses et al., 2009; Head et al,
2009; Baker et al., 2014; Anand et al., 2014; Quirós et al., 2012). It was reported that C11orf83 was degraded upon exposure to CCCP in Parkin-overexpressing cells (Chan et al., 2011). Therefore, we compared the proteolytic processing of C11orf83 and OPA1 in HeLa cells that do not overexpress Parkin under different stress conditions that induce ΔΨ loss, ATP deficiency, ROS production or ΔpH loss. We showed, by western blot analysis, that both C11orf83-V5 and OPA1 are specifically degraded upon stresses that induce ΔΨ loss (CCCP, valinomycin and carbonyl cyanide p-trifluoro-methoxyphenylhydrazone (FCCP), Figure 38 A). We performed a protease inhibitor screening to identify the enzyme(s) responsible for C11orf83 degradation. C11orf83-V5 degradation was impaired by O-PHE (1,10-phenanthroline), an inhibitor of metalloproteases, and to a lesser extent by DCI (4-dichloroisocoumarin), an inhibitor of serine proteases (Figure 38 B).

Since OMA1 is known to cleave OPA1 upon CCCP-induced stress, we downregulated OMA1 expression using siRNA and analyzed the proteolysis of C11orf83-V5 in the presence or absence of CCCP. In the absence of CCCP, the OMA1 siRNA did not modify the C11orf83-V5 protein level. However, upon CCCP stress, we observed that OMA1 downregulation was
correlated with a reduction in C11orf83-V5 degradation (Figure 39 A). The residual C11orf83-V5 proteolysis might be due to residual OMA1 protein (around 20%) unaffected by the siRNA treatment or to another metalloprotease or to DCI-sensitive serine proteases as hinted by the protease inhibitor screening assay. The same experiment performed on endogenous C11orf83 instead of overexpressed C11orf83-V5 confirmed that C11orf83 is a target of OMA1 upon mitochondrial depolarization (Figure 39 B). In contrast to data reported by Head and coll., we were not able to recover the long OPA1 isoforms with siRNA against OMA1 (Head et al, 2009). This observation can be explained by the incomplete OMA1 downregulation and/or by the absence of L-OPA1 overexpression in our experiment. In addition, as several groups have also identified that L-OPA1 is constitutively cleaved by another metalloprotease, YME1L (Song et al, 2007; Griparic et al, 2007; Anand et al, 2014; Stiburek et al, 2012), we decided to analyse the potential cleavage of C11orf83 by this protease in the presence or absence of CCCP (Figure 39 C). In both conditions, YME1L siRNA did not modify the protein level of C11orf83. This results suggested that YME1L is not involved in the constitutive or stress-induced cleavage of C11orf83. Altogether, these data confirm that C11orf83, like OPA1, plays a key role in cristae structure maintenance by allowing proper assembly of the bc1 complex and RSC stabilization, and that it may be regulated by OMA1 activity.
Figure 39: C11orf83 is cleaved by OMA1 under depolarizing conditions and not by YME1L

A) HeLa cells transfected with C11orf83-V5 and B) wild-type HeLa cells were treated with control siRNA or siRNA against OMA1. 48 h after siRNA transfection, cells were incubated with or without CCCP and cell lysates were analyzed by immunoblotting, after SDS-PAGE, using antibodies against OMA1, OPA1 and V5 tag or C11orf83. GAPDH was used as a loading control. The two bands detected by the antibody against OPA1 in the absence of CCCP correspond to short and long OPA1 isoforms. The degradation of endogenous C11orf83 under CCCP treatment (lane 3) is prevented by the depletion of OMA1 (lane 4).

C) YME1L did not cleave C11orf83 under stress conditions. 48 h after siRNA transfection, wild-type HeLa cells were incubated with or without CCCP and cell lysates were analyzed by immunoblotting, after SDS-PAGE, using antibodies against YME1L and C11orf83. GAPDH was used as a loading control. The endogenous C11orf83 is cleaved under CCCP treatment with (lane 4 and 6) and without YME1L (lane 5).
Discussion and Perspectives
Mitochondrial respiration, which generates pH and electric gradients used by the ATP synthase to drive the ATP synthesis, is supported by the four ETC complexes. OXPHOS complexes are composed of proteins encoded by nuclear and mitochondrial genomes. The biogenesis of ETC has been studied extensively over the past years and requires additional nuclear-encoded proteins called assembly factors (Ghezzi & Zeviani, 2012). The organization of the OXPHOS complexes into larger supramolecular structures called RSC is now assumed. However, the RSC assembly mechanism is still unclear and the identification of accessory proteins which facilitate RSC formation and stabilization could help to understand it. In this study, we identified C11orf83 as a new bc1 complex assembly factor and provided new insights into the bc1 complex biogenesis and RSC formation.

I. C11orf83 is a mitochondrial protein

Initially, C11orf83 had been predicted as a secreted protein following bioinformatic analysis and large scale experimental study which identified a potential signal peptide of 23 amino acids (Clark et al., 2003; Zhang and Henzel, 2004). However, C11orf83 was found among the membrane proteins in a proteomic study of crude mitochondria extracts from HeLa S3 cells (Catherman et al., 2013). We showed that C11orf83 was not a secreted protein and confirmed its mitochondrial localization by confocal microscopy and subcellular fractionation. Alkaline extraction and proteinase K assays indicated that C11orf83 is an integral IM protein with its C-terminus facing the IMS. Our results are supported by the recent work of Wanschers and coll. which reported a similar mitochondrial localization and topology for C11orf83 (Wanschers et al, 2014).

Nuclear-encoded mitochondrial proteins contain, in their aa sequence, information necessary and sufficient to direct them to the mitochondria. Many mitochondrial precursor proteins have a cleavable sequence at the N terminus, also called presequence. Classically, these presequences form an amphiphilic α-helical structure and are enriched in positively charged aa (von Heijne, 1986). The net positive charges contained in the pre-sequence are known to be important for interactions with negatively charged phospholipid headgroups (von Heijne, 1986; Paschen & Neupert, 2001). However, some proteins do not carry cleavable presequence and instead contain internal targeting signals (Paschen & Neupert, 2001). By SDS-
PAGE analysis, we detected C11orf83 at 10 kDa, corresponding to its full predicted length, suggesting that C11orf83 harbors an integral mitochondrial targeting sequence. Using a secondary structure prediction program (PSIPRED), we identified that the N-terminal part of C11orf83 (aa 1-23), previously predicted as a potential signal peptide, displays a potential amphipathic α-helix with two basic residues at positions 5 and 6. We further confirmed that this N-terminal part is responsible for the mitochondrial targeting and anchoring of C11orf83. Additionally, we demonstrated that the two basic residues at positions 5 and 6 are especially important to the correct mitochondrial localization of C11orf83.

II. C11orf83 is a new human bc1 complex assembly factor

To characterize this new mitochondrial protein, we engineered stable C11orf83-deficient cell lines and monitored mitochondrial functions. We showed that the loss of C11orf83 induced a decreased of ATP level and an impaired respiration. Although the enzymatic activities of the five respiratory complexes were found to be affected by this depletion, C11orf83 was shown to be specifically required for the bc1 complex assembly. In C11orf83-deficient cells, we observed a clear defect of the fully assembled bc1 complex combined to a light decrease of the I/II₂/IV RCS and a more pronounced diminution of the RSC III₂/IV level. In addition, C11orf83-knock down cells presented an accumulation of lower-molecular weight intermediates containing CYC1 (<100 kDa), but not UQCRCl and UQCRB. These intermediates could be aggregates of unassembled CYC1 or unassembled CYC1-subcomplexes. In yeast, Cyc1p was first proposed to be associated in subcomplex with Qcr6p and Qcr9p, until data suggested interaction with Cor1p and Cor2p. If our CYC1-intermediates are subcomplexes, the absence of UQCRCl (Cor1p homolog) in these CYC1-intermediates would support the older bc1 assembly model. It would be interesting to perform an immunoblotting against UQCRH (Qcr6p), UQCR10 (Qcr9p) and UQCR2 (Cor2p) to elucidate which type of intermediates is accumulated. However, independently from their composition, the presence of these low-molecular weight intermediates suggested the involvement of C11orf83 in an early step of bc1 assembly. We showed also that the loss of C11orf83 induced a diminution of UQCRB and UQCRQ protein levels. As these proteins compose the early bc1 core with MT-CYB, our data suggested that the loss of C11orf83 leads to the destabilization of the early core complex preventing the incorporation of CYC1 and/or CYC1-subcomplex. In
agreement with its role in the bc1 assembly, we demonstrated that C11orf83 interacts specifically with the bc1 complex.

Altogether, our work indicates that C11orf83 is a new human bc1 assembly factor assisting early steps of bc1 biogenesis, probably by stabilizing the early core complex (MT-CYB/UQCRB/UQCRQ). Our results are supported by recent data obtained by Wanschers and coll. which identified a missense mutation in the coding sequence of C11orf83 gene (c.59T>A) that results in a non-conservative aa change (p.Val20Glu). The mutation leads to an unstable mutant C11orf83 protein which becomes undetectable in the patient’s mitochondria and causes a bc1 complex deficiency. (Wanschers et al, 2014). They showed in fibroblast of patient that C11orf83, that they renamed UQCC3 (Ubiquinol-cytochrome-c reductase complex assembly factor 3), is necessary for MT-CYB stability and incorporation in the bc1 complex. Based on this data, it would be interesting to monitor MT-CYB synthesis and its incorporation in the bc1 complex by pulse-chase experiments in our C11orf83/UQCC3-deficient cell lines. Mitochondrial-encoded proteins would be radiolabeled and analyzed by BN and 2D BN/SDS-PAGE.

III. C11orf83/UQCC3 could be the human functional analogue of Cbp4p

Since the bc1 complex structure is well conserved among organisms, we could speculate that most assembly factors would be similar in human and lower organisms. Until now, TTC19 is the sole human bc1 assembly factor without a known yeast homolog. In yeast, seven proteins have been identified as involved in bc1 assembly, from the exit from the newly synthesized Cobp of the mitochondrial ribosomal tunnel until the incorporation of the last subunit Rip1p in to the mature bc1 complex: Cyt2p (Zollner et al, 1992), Cbp3p (Gruschke et al, 2012), Cbp6p (Gruschke et al, 2012), Mzm1p (Atkinson et al, 2011), Bcs1p (Wagener & Neupert, 2012), Bca1p (Mathieu et al, 2011) and Cbp4p (Crivellone, 1994; Hildenbeutel et al, 2014). Among these yeast assembly factors, only Bca1p and Cbp4p still lack human homologs.

No obvious homolog of C11orf83/UQCC3 in yeast could be detected by Psi-BLAST analysis. Both Bca1p and Cbp4p are involved in the early steps of bc1 assembly (Hildenbeutel et al, 2014; Gruschke et al, 2011; Mathieu et al, 2011) and both are IM proteins with the same topology as C11orf83/UQCC3 (Mathieu et al, 2011; Crivellone, 1994; Kronekova & Rödel, 2005).
Therefore, we suspected that C11orf83/UQCC3 could be the functional homolog of either Bca1p or Cbp4p. Bca1p is a large protein of 66 kDa with RCC1/BLIP1I motifs (Regulator of Chromosome Condensation; β-lactamase inhibitor protein-I) at its C-terminus (Mathieu et al., 2011), which may form β-propeller folds that can bind protein ligands (Renault et al., 1998). According to these latest features, the probability that Bca1p is the yeast analogue of C11orf83/UQCC3 is weak. Analysis of data obtained by Mathieu and coll. supported this idea (Mathieu et al., 2011). Indeed, no interaction between Bca1p and the bc1 complex was reported; Bca1p could not be immunoprecipitated with any bc1 subunit, and Bca1p did not migrate at a similar molecular weight than the bc1 complex (Mathieu et al., 2011). Moreover, although the loss of Bca1p induced a defect in bc1 assembly, authors noted that no accumulation of bc1 intermediates could be revealed, despite a long exposition time of immunoblotting, unlike what we observed with the loss of C11orf83/UQCC3 (Mathieu et al., 2011).

Despite a longer amino acid sequence than C11orf83/UQCC3 (147 versus 93 residues) and a low aa sequence identity between C11orf83/UQCC3 and Cbp4p (24%), Cbp4p could be the yeast functional homolog of C11orf83/UQCC3 (Figure 40). Indeed, experimental observations reported for Cbp4p are similar as what we showed for C11orf83/UQCC3. For example, Δcbp4 mutant yeast strains and C11orf83/UQCC3-knock down human cells display an identical accumulation of CYC1/Cyc1p-containing subcomplexes, and similarly reduced steady-state levels of UQCRB/Qcr7p and UQCRQ/Qcr8p (Kronekova & Rödel, 2005). As for C11orf83/UQCC3, the N-terminal part of Cbp4p has been found to be involved in mitochondria membrane targeting and anchoring (Crivellone, 1994). To note, only one of the two positively charged amino acids that we identified as essential for the mitochondrial localization of C11orf83/UQCC3, are conserved in the yeast and Danio rerio proteins (Figure 40, residues highlighted in blue). It is tempting to speculate that this positively charged aa would be sufficient for the mitochondrial localization of both proteins. To verify this hypothesis, we could analyse the cellular localization of K6A-N23-GFP (corresponding to the mutated N-terminal sequence of C11orf83/UQCC3 N-terminally fused with the GFP protein), by confocal microscopy.
Figure 40: Sequence alignment of human C11orf83/UQCC3 with its orthologs and the potential yeast homolog Cbp4p. Q6UW78 (Human), Q148G8 (Bovine), Q8K2T4 (Mouse), B7ZQL6X (XENLA, Xenopus laevis), E9QEL2 (DANRE, Danio rerio), P37267 (Yeast Cbp4p). The location of the Val20Glu mutation identified by Wanschers and coll. is indicated by an arrow. Predicted α-helices (black cylinders) and β-strand (grey arrow) for the metazoan sequences are displayed above the alignment unlike the prediction structure for the yeast sequence is presented below the alignment. The conserved RK motif in mammalian is highlighted by a red box. The positively charged aa conserved up to yeast is highlighted in blue. Both putative GXXXG motifs in the transmembrane segment of C11orf83/UQCC3 are highlighted in orange and red.

In collaboration with the complex Cbp3p–Cbp6p, Cbp4p has been shown to be involved in the maturation of Cobp. Cbp4p seems required for the formation of a stable, semi-hemylated Cobp containing the b$_1$ heme (Gruschke et al, 2012, 2011; Hildenbeutel et al, 2014). It was shown that Cbp4p is associated to the bc1 early core complex by interacting with Cobp. These data are in accordance with the bc1 early core complex stabilizing function that we identified for C11orf83/UQCC3. This analogy has also been suggested in the recent paper of Wanschers and coll (Wanschers et al, 2014). To confirm that Cbp4p and C11orf83/UQCC3 share a similar function, it would be necessary to demonstrate that C11orf83/UQCC3 specifically interacts with MT-CYB. For this purpose, we could use the protocol of single step affinity purifications which has been set up by Turcker and coll. to identify the interaction of UQCC1 with the $^{35}$S metabolically labeled translated MT-CYB in HEK293 cells (Tucker et al, 2013)

Some differences exist between C11orf83/UQCC3 and Cbp4p and could explain why Wanschers and coll. failed to complement Δcbp4 mutant yeast strains with C11orf83/UQCC3 (Wanschers et al, 2014). Although Kronekova and Rödel observed that Cbp4p-containing complexes could co-migrate with the bc1 complex and weakly with the II$_2$/IV RSC (Kronekova & Rödel, 2005), more recent work indicate that Cbp4p would be released from the bc1 core complex before the incorporation of Cor1p and Cor2p (Gruschke et al, 2012). Cbp4p would not be involved in the RCS stabilization, in contrast to what we propose for C11orf83/UQCC3.
Additionally, despite that Cbp4p has been found interacting with Cbp3p-Cbp6p-Cobb, Wanschers et coll. could not observe any interaction between the three human proteins, C11orf83/UQCC3, UQCC1 (Cbp6p) and UQCC2 (Cbp3p), using 2D BN/SDS-PAGE analysis or tandem affinity purification (TAP)-tag experiments. However, they observed an instability of C11orf83/UQCC3 when UQCC1 or UQCC2 are depleted, which supports the possibility of a functional interdependence between these proteins (Wanschers et al, 2014). These differences of behaviour between the yeast and human proteins could be due to their low level of sequence similarity. Such non-overlapping properties would join the growing list of evolutionary differences that are known to exist between the yeast and mammalian ETC systems such as the difference in the subunit composition of ETC complexes (the pre-sequence of UQCRFS1 as an additional subunit in mammals) or the lack of CI in *Saccharomyces cerevisiae*. Thanks to our findings and the work of Wanschers and coll., we can propose the following model for human bc1 assembly (*Figure 41*). This model cumulates informations obtained by biochemical analysis of cells derived from patients suffering from mitochondrial complex III deficiency, and informations obtained by homology with the available data for yeast. In our model, we propose that C11orf83/UQCC3 is associated to MT-CYB based on data obtained in yeast for Cbp4p and due to the fact that C11orf83/UQCC3 is required for stability of MT-CYB and the early core complex (Wanschers et al, 2014; Gruschke et al, 2012; Hildenbeutel et al, 2014).
Figure 41: New model for human bc1 assembly. Together with the work of Wanschers and coll., we propose a model of human bc1 assembly where C11orf83/UQCC3 is required for the stabilization of MT-CYB and assists the incorporation of the early core complex in the bc1 complex (Wanschers et al., 2014). Contrarily to their yeast homologues, UQCC1 and UQCC2 seems to be released from MT-CYB before the insertion of C11orf83/UQCC3, because Wanschers and coll. could not observe any interaction between these three proteins. The interaction of C11orf83/UQCC3 with MT-CYB and the early core complex is based on data obtained in yeast with Cbp4p (Gruschke et al., 2012; Hildenbeutel et al., 2014), because it has not yet been demonstrated in human. Of note, the position of C11orf83/UQCC3 within the bc1 complex is speculative. Adapted from (Fernández-Vizarra & Zeviani, 2015).

Even if C11orf83/UQCC3 seems to be the functional analogue of Cbp4p, the molecular mechanisms used by C11orf83/UQCC3 to stabilize the bc1 core complex and then assist the bc1 complex assembly are still unknown. To decipher these mechanisms, we propose two avenues of work detailed in the following section.
IV. How could C11orf83/UQCC3 assist the bc1 assembly?

IV.1. CL-binding property of C11orf83/UQCC3

We have shown that C11orf83/UQCC3 was able to bind to CL and to its precursor PA (Schlame & Haldar, 1993). CL is an important IM phospholipid involved in the stabilization of individual ETC complexes and more precisely the bc1 enzyme (Slavotinek & Biesecker, 2000; Yu & Yu, 1980; Wenz et al, 2009). It was estimated that the bc1 complexes are associated to 8-9 molecules of tightly bound CL (Hayer-Hartl et al, 1992). Among the CL identified in yeast and bovine bc1 complexes, four interact with at least one protein of the early core complex (MT-CYB/UQCRB/UQCRQ, cf Table 2). These CL-binding sites are critical for the structural integrity of the bc1 complex as suggested by the destabilization of bc1 complex and the reduced amount of Cobp observed after site-directed mutagenesis on some CL-binding sites (Palsdottir & Hunte, 2004; Lange et al, 2001). In the context of these data, we could speculate that the ability of C11orf83/UQCC3 to bind CL contributes to the structural stabilization of the early core complex and the bc1 enzyme.

The identification of the CL-binding site of C11orf83/UQCC3 would allow us to work on this hypothesis. We found that the CL-binding of C11orf83/UQCC3 did not require its N-terminal part, responsible for IM targeting and anchoring, but both α-helices of the IMS part, indicating that they provide a unique tertiary structure suitable for interaction with CL. Similar observations were reported for the truncated pro-apoptotic protein tBID, whose CL-binding domain encompasses three helices, H4, H5 and H6 (Lutter et al, 2000). Several studies showed that some aa, such as lysine, arginine, phenylalanine and leucine, are often involved in CL binding sites of OXPHOS complexes (Arnarez et al, 2013a; Pöyry et al, 2013; Arnarez et al, 2013b). Despite some trials, we failed to identify what specific residues are involved in CL binding. Additional structural investigations by X-ray crystallography coupled to extensive site-directed mutagenesis studies would be needed to characterize the interaction of C11orf83/UQCC3 with CL at the molecular level. Once the residues involved in the CL-binding of C11orf83/UQCC3 would be identified, we could express a mutated C11orf83/UQCC3 unable to bind CL in C11orf83/UQCC3-deficient cells and observe its effect on bc1 complex assembly.
IV.2. GXXXG motif

In membranes, protein-protein interactions are mediated mainly through transmembrane domains (Fink et al., 2012). Among conserved motifs known to drive transmembrane interactions (Fink et al., 2012), we identified two GXXXG motifs in the single-spanning transmembrane of the mammalian C11orf83/UQCC3: G15xxxG19 and G17xxxG21 (Figure 40 glycine residues highlighted in red and orange) (Russ & Engelman, 2000). The GXXXG sequence corresponds to a motif where two conserved glycine residues are separated by any three aa on a helical framework (Russ & Engelman, 2000). Thanks to the three-spacer resides, the glycine residues of the motif are presented on one face of the helix and the small nature of the side chain of glycine permits the formation of a close helix-helix interaction (MacKenzie, 1997; Russ & Engelman, 2000). The GXXXG motif has been linked to the homo- or heterodimerization of membrane proteins such as glycophorin A (Lemmon et al., 1992, 1994) or EGFR and ERBB2 (Gerber et al., 2004). Exchange of glycine residues by aa with larger side chains, such as valine, leucine, or isoleucine, interferes with the packing interface of neighboring helices and prevents dimer formation as observed for the glycophorin A (Lemmon et al., 1992). In IM of mitochondria, two subunits of the yeast ATP synthase, Atp20p and Atp21p have GXXXG motifs located in their membrane-spanning domains. These motifs are conserved until human and are involved in the dimerization/oligomerization of the yeast ATP synthase (Bustos & Velours, 2005; Arselin et al., 2003; Saddar & Stuart, 2005). Similarly, it was recently shown that GXXG motifs present in the two first transmembrane domains of the yeast Tim23p are required for the structural integrity of the TIM23 complex. Authors proposed that these GXXXG motifs are involved in Tim23p dimer formation and potentially in Tim23p-Tim17p interaction due to the presence of similar motifs in Tim17p (Demishtein-Zohary et al., 2015). Accordingly, we could speculate that one of the GXXXG motifs observed in C11orf83/UQCC3 could be involved in the structural integrity of the bc1 complex.

The first GXXG motif (G15xxxG19) is conserved in mammalian C11orf83/UQCC3 and in yeast Cbp4p (Figure 40 glycine residues in red) which therefore suggests that it is potentialy involved in the function of C11orf83/UQCC3. This is also supported by the pathogenic mutation at the valine (Val20Glu, Figure 40 black arrow) adjacent of this motif identified by Wanschers and coll (Wanschers et al., 2014). Indeed, it was already found that the residues surrounding GXXG
motifs contribute in the affinity of the helix interactions (Lemmon et al., 1992). For example, extensive site-directed mutagenesis studies revealed that the replacement of the adjacent valine (Val80 and Val84) residues of the G79xxxG83 motif of glycophorin A resulted in a decreased dimerization of glycophorin A (Lemmon et al., 1992). The analysis of the 3D structure of the dimeric transmembrane domain of glycophorin A suggested that the side chains of Val80 and Val84 form “a ridge that packs against the groove created by Gly79 and Gly83 of the opposite monomer” (MacKenzie, 1997). The replacement of the Val20 in C11orf83/UQCC3 by a negatively charged residue could prevent helix interactions and destabilize C11orf83/UQCC3.

The bc1 complex exists as a dimer, but the dimerization step of this enzyme is still unclear (Zara et al., 2009b; Conte et al., 2015). The GXXXG motifs located in the transmembrane of C11orf83/UQCC3 could promote a C11orf83/UQCC3-dimerization and then contribute to the dimerization of the bc1 complex. Another possibility would be that C11orf83/UQCC3 could stabilize a subunit of the bc1 complex by a transmembrane heterodimerization. In this respect, it is interesting to note that MT-CYB also contains a GXXXG motif in its first transmembrane. Moreover, the mutation of the first glycine of the putative GXXXG motif of MT-CYB to serine (Gly34Ser) have been associated to a mitochondrial complex III deficiency (Andreu et al., 1999). We could speculate that C11orf83/UQCC3 may assist the early step of bc1 biogenesis by stabilizing MT-CYB via a GXXXG-mediated heterodimerization.

Site-directed mutagenesis on both GXXXG motifs of C11orf83/UQCC3 (G15xxx19 and G17xxxG21) are required to investigate their potential involvement in bc1 assembly. As AXXXXA motifs have also been reported to promote interactions between transmembrane helices (Kleiger et al., 2002), glycine residues at position 15 and 17 could be turned to leucine residues, individually and at the same time. Mutated C11orf83/UQCC3 proteins could be expressed in our C11orf83/UQCC3-deficient cells and we could analyze the bc1 assembly by 2D BN/SDS-PAGE analysis. We could also perform these mutageneses in the plasmid encoding WT-N23-GFP protein (corresponding to the N-terminal sequence of C11orf83/UQCC3 N-terminally fused with the GFP protein). Therefore, we could express wild-type and mutant N23-GPF proteins and monitor the potential rescue of the bc1 assembly in C11orf83/UQCC3-deficient cells. By this way,
we would abolish the CL binding ability of C11orf83/UQCC3, and then study only the role of the transmembrane segment in bc1 assembly.

To note, glycine residues at position 15 and 17 are located in the transmembrane segment of C11orf83/UQCC3 which also correspond to its mitochondrial targeting sequence. It would then be important to control the correct IM mitochondrial localization of the C11orf83/UQCC3 proteins displaying mutation at these positions.

V. C11orf83/UQCC3 and RCS stabilization

Beside the bc1 assembly defect, we observed that the loss of C11orf83/UQCC3 also induced a significant reduction in the amounts of III2/IV and III2/IV RSC, with a more pronounced effect for the III2/IV RSC. These observations could be explained by two hypotheses. Either C11orf83/UQCC3 is involved both in bc1 assembly and in the interaction of this complex with CIV, or C11orf83/UQCC3 is involved only in bc1 assembly and the reduced level of these RCS is just a secondary consequence of the bc1 assembly deficiency. In the literature, we found studies supporting both hypothesis. Two yeast proteins Rcf1p and Rcf2p, have been reported as relevant to assembly between the bc1 complex and CIV, as well as to the maturation of the CIV (Vukotic et al., 2012; Strogolova et al., 2012; Chen et al., 2012). In contrast, according the Enríquez’s group, a true RCS chaperone would allow assembly of RSC but not of the individual complexes. Then, they proposed COX7A2L, renamed SCAFI, as a RCS assembly factor required for stable interaction between the bc1 complex and CIV (Lapuente-Brun et al., 2013).

We showed an interaction between C11orf83/UQCC3 with the bc1 complex, which is in agreement with its role in the bc1 complex assembly. In the same conditions, we did not identify any interaction between C11orf83/UQCC3 and CIV. This could be interpreted as evidence against an involvement of C11orf83/UQCC3 in the interaction between the bc1 complex and CIV. However, we cannot exclude a weak interaction of C11orf83/UQCC3 with CIV, because we observed that C11orf83/UQCC3 co-migrated not only with bc1 complex and III2/IV RSC in native gels, but also with CIV and the dimer of CIV. Similar observations have been reported for Rcf1p, which stably interacts with CIV to assist its assembly, but also weakly binds to the bc1 complex to stabilize the III2/IV2 RSC (Vukotic et al., 2012; Chen et al., 2012).
Furthermore, we detect C11orf83/UQCC3 in the III$_2$/IV RSC but not in the I/III$_2$/IV RSC. We could speculate that the presence of CI stabilizes the interaction between the bc1 complex and CIV rendering C11orf83/UQCC3 stabilizing function useless. In the literature, two models for I/III$_2$/IV assembly were proposed: Enríquez’ model where formation of RCS would be subsequent to the assembly of the three individual complexes (Figure 17 A) (Acín-Pérez et al, 2008) or Ugład’s model where a CI-intermediate is used as a scaffold for the incorporation of bc1 complex and CIV subunits and assembly intermediates (Figure 17 B) (Moreno-Lastres et al, 2012). Our observation could support Ugład’s model because in this context the formation of III$_2$/IV RSC is independent of I/III$_2$/IV, which could explain the presence of C11orf83/UQCC3 only in III$_2$/IV (Figure 42 B). As we did not find a similar behavior in the literature we can not exclude a second option more in agreement with Enríquez’s model. Indeed, we could propose that C11orf83/UQCC3 is involved in the stabilization of the III$_2$/IV RSC and be removed when this smaller RSC associates to CI. The formation of the I/III$_2$/IV RSC could stabilize the interaction between the bc1 complex and CIV, rendering C11orf83/UQCC3 stabilizing function redundant, and leading to the elimination of this protein from the RSC (Figure 42 A). In the literature, the CI assembly chaperone mimitin (NDUFAF2) is also transiently used for a specific step of assembly and then released to allow assembly completion (Lazarou et al, 2007).
Figure 42: Integration of C11orf83/UQCC3 in the two current models proposed for RCS assembly. During all this study, we detected C11orf83/UQCC3 in the bc1 complex and in III2/IV RSC, but not in IIIb/IV RSC A) Model proposed by the group of Enríquez where the formation of RSC occurs after the preliminary assembly of each complexes (Acín-Pérez et al., 2008) B) Model proposed by Uglade’s group suggesting the use of a CI-intermediate scaffold for the incorporation of the bc1 complex and CIV subunits (Moreno-Lastres et al., 2012). Adapted from (Moreno-Lastres et al., 2012; Porras & Bai, 2015).

CL are enriched in RSC and are involved in their formation and stabilization (McKenzie et al., 2006; Pfeiffer et al., 2003; Althoff et al., 2011; Bazán et al., 2013). Several CL-binding sites were identified in the bc1 complex (Lange et al., 2001; Amarez et al., 2013b) and in CIV (Amarez et al., 2013a) and it was proposed that CL could form bridges between these complexes to stabilize the III2/IV RSC (Amarez et al., 2013a). Accordingly, the ability of C11orf83/UQCC3 to bind CL could contribute to stabilize the III2/IV RSC.

VI. C11orf83/UQCC3 is involved in cristae maintenance

Besides the reduced RSC levels, we showed that C11orf83/UQCC3 loss also induced a disorganization of the mitochondrial ultrastructure accompanied by changes in the acyl chain composition of CL. Similar results were observed in the patients suffering from Barth syndrome, where the loss of TAZ, an enzyme involved in CL maturation, causes an accumulation of
monolyso-CL (a remodeling intermediate) and an altered acyl chain composition of CL (Houtkooper et al., 2009b; Schlame et al., 2003). This abnormal composition of CL is combined to a severe defect in cristae organization (Acehan et al., 2007). The acyl chain composition of CL differs between organisms and even between cell types and tissues in a single organism and seems important to the cristae maintenance and/or to the adaptation of the cell to its environment (Hoch, 1992; Schlame et al., 2005). We cannot exclude that C11orf83/UQCC3 could modulate the CL remodeling pathway. However according to our data about the function of C11orf83/UQCC3, it is most likely that the modification of CL fatty acid composition represents an IM adaptation to the defects in mitochondria functions and structure caused by the depletion of C11orf83/UQCC3.

The mitochondrial ultrastructure impairment observed in C11orf83/UQCC3-deficient cells may also explain the reduced enzymatic activity of all the ETC complexes and the higher sensitivity to actinomycin D-induced apoptosis as reported for OPA1-depleted cells (Kushnareva et al., 2013; Olichon et al., 2003). Since cristae membranes are enriched in OXPHOS complexes and ensure optimal condition for ATP synthesis (Gilkerson et al., 2003; Kay et al., 1985; Demongeot et al., 2007), a disorganized cristae is linked to a reduced respiratory efficiency (Cogliati et al., 2013). Additionally, the proapoptotic protein CYC is predominantly stored in intracristal space (Scorrano et al., 2002) and a remodeling of the cristae is required to allow its release during apoptosis (Scorrano et al., 2002; Frezza et al., 2006; Yamaguchi et al., 2008). In condition of disorganized mitochondria, CYC release is facilitated leading to an enhanced sensitivity to apoptosis (Arnoult et al., 2005). Based on our observations of C11orf83/UQCC3-deficient cells, it would be interesting to monitor the kinetics of CYC release under apoptosis-stimuli. For this purpose, isolated mitochondria from C11orf83/UQCC3-downregulated cells would be treated with recombinant truncated BID, an outer membrane permeabilizing molecule, and the release of CYC could be evaluated by western blot and ELISA.

**VII. C11orf83/UQCC3 is a target of OMA1**

We demonstrated that OMA1 metalloprotease mediates the cleavage of C11orf83/UQCC3 in response to mitochondrial depolarization. This metalloprotease is already known to be involved in the constitutive and stress-mediated cleavage of L-OPA1 isoforms (Ehses et al., 2009; Head et al., 2009; Baker et al., 2014; Anand et al., 2014; Quirós et al., 2012).
Under stress conditions, OMA1 is activated and triggers the complete conversion of L-OPA1 isoforms into the short isoforms leading to the inactivation of OPA1. These molecular events coordinate mitochondrial fragmentation which can lead to apoptosis (Ehses et al, 2009; Head et al, 2009; Baker et al, 2014; Anand et al, 2014). Among the stresses which induce OMA1 activation and OPA1 cleavage, ATP depletion was reported (MacVicar & Lane, 2014; Baricault et al, 2007; Head et al, 2009; Zhang et al, 2014; Baker et al, 2014). However, we observed that C11orf83/UQCC3 was not cleaved in the presence of oligomycin. We can speculate that our experimental conditions (4 µM of oligomycin during 3 h in the presence of glucose) did not allow a sufficient ATP depletion to activate OMA1. Indeed, in HeLa cells treated with 2.5 µM of oligomycin during 4 h, OMA1 is only slightly activated (Zhang et al, 2014), and we can suppose that this level of activation would not be sufficient to a proper cleavage of C11orf83/UQCC3 and OPA1. An analysis of experimental conditions used in the literature revealed a different sensitivity of OMA1-mediated cleavage of OPA1 to oligomycin in the different cell lines (Table 4). In MEF cells, weak concentrations of oligomycin (1-2 µM) during a short time exposure (1-2 h) were sufficient to observe a OPA1 cleavage (Baker et al, 2014; Ehses et al, 2009). In contrast, in hTERT-RPE1 cells, OMA1 did not cleave OPA1 in presence of 10 µM of oligomycin in a medium with glucose despite 6 h of exposure (MacVicar & Lane, 2014). In these cells, the OPA1 cleavage can be observed only in the presence of oligomycin in a glucose-free medium (MacVicar & Lane, 2014). In this way, it would be important to monitor the level of ATP in our experimental conditions and test the cleavage of C11orf83/UQCC3 by OMA1 in more stringent conditions such as the use of glucose-free medium.
Table 4: Experimental conditions used in the literature to observe OMA1 activation or L-OPA1 cleavage under ATP depletion

MEF: Mouse embryonic fibroblasts; hTERT-RPE human telomerase-immortalized retinal pigment epithelium

It was also recently shown that OMA1 is specifically activated by apoptosis stimuli in a Bax- and Bak-dependent fashion and that the subsequent OMA1-mediated cleavage of OPA1 triggers the remodeling of mitochondrial cristae. This remodeling allows the release of CYC stored inside the cristae (Jiang et al., 2014). As we observed that the loss of C11orf83/UQCC3 induced a disorganization of the cristae associated to an enhanced sensitivity to apoptosis, the cleavage of C11orf83/UQCC3 by OMA1 may contribute to the mechanism triggering apoptosis under stress (Figure 43).
Figure 43: The proposed role of stress-induced cleavage of C11orf83/UQCC3 by OMA1. Under stress stimuli, OMA1 is activated and triggers the complete conversion of L-OPA1 to S-OPA1 (Ehses et al, 2009; Head et al, 2009; Baker et al, 2014; Anand et al, 2014). In parallel, the same metalloprotease cleaves C11orf83/UQCC3, which becomes undetectable in mitochondria extracts. Both the loss of C11orf83/UQCC3 and the cleavage of OPA1 cause cristae remodelling leading to a higher sensitivity to apoptosis. Therefore, the OMA1-mediated cleavage of these two proteins under stress conditions can contribute to the elimination by apoptosis of cells with damaged mitochondria.

It would be interesting to confirm the physiological significance of the OMA1-mediated cleavage of C11orf83/UQCC3. For this purpose, we first need to set up experiments where this cleavage is inhibited. As OMA1 targets several proteins under stress condition the specific inhibition of the OMA1-cleavage of C11orf83/UQCC3 is not possible using siRNA against OMA1 or with anti-metalloprotease chemical inhibitors. Therefore, we need to generate stable cell lines in which endogenous C11orf83/UQCC3 would be replaced by a mutated form of this protein resistant to the OMA1 cleavage. To this end, the identification of the OMA1 cleavage site in C11orf83/UQCC3 is required and could be obtained by combining alanine scanning mutagenesis and mass spectrometry analysis.

In addition, Oma1−/− mice exhibit a marked obesity with metabolic alterations, reduced energy expenditure and altered thermogenic response, indicating that OMA1 is a key regulator of metabolic homeostasis (Quirós et al, 2012). Moreover, it was shown that OMA1 is activated in vivo by renal ischemia and that the OMA1-mediated OPA1 cleavage contributes to apoptosis and kidney damages (Xiao et al, 2014). As C11orf83/UQCC3 is also a downstream effector of OMA1, we could suspect a similar cleavage of C11orf83/UQCC3 in kidney tissues of these mice.
VIII. Mutation in C11orf83/UQCC3 causes a mitochondrial complex III deficiency

We demonstrated that C11orf83/UQCC3 is an assembly factor of the bc1 complex which could be involved in the maintenance of metabolic homeostasis because of its sensitivity to OMA1. Moreover, we observed that its depletion caused severe mitochondrial dysfunctions in vitro. The importance of C11orf83/UQCC3 in vivo was confirmed by Tang and coll., which performed the knock-out of C11orf83/UQCC3 gene in mice and observed an embryonic death of KO mice (Tang et al., 2010). More importantly, the identification of a deleterious mutation in C11orf83/UQCC3 in a patient diagnosed with a bc1 complex deficiency highlighted the essential role of C11orf83/UQCC3 in vivo (Wanschers et al., 2014). This mutation leads to an unstable protein that is undetectable in the mitochondria from patient’s fibroblasts. Due to the loss of active C11orf83/UQCC3, this patient suffers of eating and sleeping difficulties, muscular weakness, increased fatigability, delayed growth and psychomotor development, since her birth (Wanschers et al., 2014).

Among the mitochondrial diseases due to OXPHOS disorders, mitochondrial complex III deficiency is the least frequently diagnosed one (Janssen et al., 2006; Diaz et al., 2011; Fernández-Vizarra & Zeviani, 2015). The principal reasons are that this deficiency is characterized by a wide range of symptoms and disease phenotypes and most patients with complex III deficiency die in early childhood (Moslemi & Darin, 2007). Additionally, in most cases, the genetic and molecular mechanisms responsible for the bc1 defect remain unknown. This is likely due to a lack of understanding of the bc1 complex assembly process and of the required assembly factors (Janssen et al., 2006; Diaz et al., 2011; Fernández-Vizarra & Zeviani, 2015). Pathological mutations have already been reported for several assembly factors, BCS1L (Fernández-Vizarra & Zeviani, 2015; de Lonlay et al., 2001), TTC19 (Ghezzi et al., 2011; Morino et al., 2014; Kunii et al., 2015; Atwal, 2014), LYRM7/MZML1 (Invernizzi et al., 2013) and UQCC2 (Tucker et al., 2013). For two of them (TTC19 and BCS1L), different mutations have been reported leading all to OXPHOS disorders (Fernández-Vizarra & Zeviani, 2015). It is likely that new patients with C11orf83/UQCC3 mutations will be identified in the near future. An extended
C11orf83/UQCC3 mutational screening, by traditional or next-generation approaches (Carroll *et al*, 2014), to all patients with mitochondrial complex III deficiency should be considered.

Together with the work of Wanschers and coll., the present study provides new insights into the bc1 complex biogenesis and therefore contributes to progress in the diagnosis and understanding of mitochondrial complex III deficiencies. That is why it is important to continue to decipher the human bc1 complex assembly by identifying partners of C11orf83/UQCC3 and new bc1 assembly factors.
Material and Methods
## I. Material

### I.1. Reagents

#### I.1.1. Proteinase K assay

<table>
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<tr>
<td>Saponin</td>
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<tr>
<td>Proteinase K</td>
<td>Macherey Nagel (Düren, Germany)</td>
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#### I.1.2. Detergents

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<tr>
<td>Digitonin</td>
<td>EMD Millipore (Darmstadt, Germany)</td>
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#### I.1.3. Mitochondrial stress-inducers

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<td>Enzo Life Sciences</td>
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| Carbonyl cyanide p-trifluoro-
  methoxyphenylhydrazone (FCCP)     | Enzo Life Sciences        |
| Valinomycin                         | Enzo Life Sciences        |
| Oligomycin A                        | Enzo Life Sciences        |
| Carbonyl cyanide m-chlorophenyl
  hydrazone (CCCP)                   | Sigma-Aldrich             |
| Rotenone                            | Sigma-Aldrich             |
| Dimethylsulfoxide (DMSO)            | Sigma-Aldrich             |

#### I.1.4. Protease inhibitors

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<td>E-64-D</td>
<td>Enzo Life Sciences</td>
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<td>Pepstatin A</td>
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<td>Phenylmethanesulfonyl fluoride (PMSF)</td>
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<td>3,4-dichloroisoucoumarin (DCI)</td>
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<td>1,10-phenanthroline (O-PHE)</td>
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I.1.5. Apoptosis assay

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<td>Z-VAD-FMK</td>
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I.1.6. Spectrophotometric assays for respiratory complexes

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I.1.7. Recombinant GST proteins expression and purification

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I.2. Mammalian cells culture and transfection

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### I.3. Antibodies

#### I.3.1. Primary antibodies

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<td>AbD Serotec MCA2892GA</td>
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Material and Methods

I.3.2. Secondary antibodies

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<td>Santa Cruz Biotechnologies sc-2770</td>
</tr>
<tr>
<td>Goat anti-mouse Alexa fluor 488</td>
<td>IF, 1/400</td>
<td>Life Technologies A-11001</td>
</tr>
<tr>
<td>Goat anti-rabbit Rhodamine</td>
<td>IF, 1/400</td>
<td>Life Technologies R-6394</td>
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</table>

Ig: Immunoglobulin; WB: Western blot; IF: Immunofluorescence; HRP: horseradish peroxidase.

I.4. Molecular weight markers

<table>
<thead>
<tr>
<th>Name</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>BenchTop 1 kb DNA Ladder</td>
<td>Promega (Fitchburg, USA)</td>
</tr>
<tr>
<td>BenchTop 100 bp DNA Ladder</td>
<td>Promega</td>
</tr>
<tr>
<td>Prestained Protein Ladder</td>
<td>BioRad (Hercules, USA)</td>
</tr>
</tbody>
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### I.5. Kits and other materials

<table>
<thead>
<tr>
<th>Name</th>
<th>Supplier</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNaseqy</td>
<td>Qiagen (Venlo, Netherlands)</td>
<td>For RNA extraction</td>
</tr>
<tr>
<td>Quick start Bradford</td>
<td>BioRad</td>
<td>For protein concentration determination</td>
</tr>
<tr>
<td>bc1 complex immunocapture kit</td>
<td>Abcam (ab109800)</td>
<td>For immunoprecipitation of the bc1 complex</td>
</tr>
<tr>
<td>CIV immunocapture kit</td>
<td>Abcam (ab109801)</td>
<td>For immunoprecipitation of CIV</td>
</tr>
<tr>
<td>ATP bioluminescence HS II</td>
<td>Roche Applied Bioscience</td>
<td>For ATP concentration determination</td>
</tr>
<tr>
<td>MitoXpress kit</td>
<td>Luxel Biosciences Ltd (Cork, Ireland)</td>
<td>For oxygen consumption measurement</td>
</tr>
<tr>
<td>Gateway cloning kit</td>
<td>Life Technologies</td>
<td>For cloning DNA insert into Gateway vectors</td>
</tr>
<tr>
<td>Annexin V- APC staining kit</td>
<td>BD Biosciences</td>
<td>For apoptosis detection using flow cytometry</td>
</tr>
<tr>
<td>Commercial panel of human tissue cDNA</td>
<td>Clontech Reference, 636753</td>
<td>For determination of tissue distribution and relative abundance of specific transcripts</td>
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</table>

### I.6. shRNA plasmids and siRNA

#### I.6.1. shRNA plasmids

The C11orf83 shRNA plasmids were built using C11orf83 shRNA primers that were designed according to the manufacturer’s instructions (Figure 44, Biosettia, San Diego, USA). These sequences were inserted in pRNAi-H1-puro plasmid according to manufacturer’s instructions. A negative control pRNAi with sh-lacZ insertion was provided by manufacturer.

![Schematic representation of the C11orf83 shRNA primers inserted in pRNAi-H1-puro plasmid.](image)
I.6.2. siRNA

OMA1 expression was downregulated by 25 pmol of siRNA (s41775, Life Technologies) and YME1L with 25 pmol of siRNA (21077, Life Technologies). As control siRNA, the silencer select negative control #1 was used at 25 pmol (4390843, Life Technologies).

I.7. Primers

<table>
<thead>
<tr>
<th>Nº</th>
<th>Primer name</th>
<th>Sequence</th>
<th>Description</th>
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<tr>
<td>1</td>
<td>C11orf83-F</td>
<td>CACCATGGATTCCTTGCGGAAAATGCTGATCTC</td>
<td>For insertion in pENTR™/SD/D-TOPO</td>
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<tr>
<td>2</td>
<td>C11orf83-R (without STOP)</td>
<td>CGGTGACCTCCCGCGGCG</td>
<td>For insertion in pENTR™/SD/D-TOPO</td>
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<td>3</td>
<td>GAPDH-F</td>
<td>TGAAGGTCGGTGCTAAGGATTTGCG</td>
<td>PCR primer</td>
</tr>
<tr>
<td>4</td>
<td>GAPDH_R</td>
<td>CATGTAAGGCCATGAGGTCCACCA</td>
<td>PCR primer</td>
</tr>
<tr>
<td>5</td>
<td>N23_F</td>
<td>CACCATGGATTCCTTGCGGAAAATGCTGATCTC</td>
<td>For insertion in pENTR™/SD/D-TOPO</td>
</tr>
<tr>
<td>6</td>
<td>N23_R</td>
<td>CGGTGACCTCCCGCGGCG</td>
<td>For insertion in pENTR™/SD/D-TOPO</td>
</tr>
<tr>
<td>7</td>
<td>C11orf83-F (without ATG)</td>
<td>CACCGATTCCTTGCGGAAAATGCTGATCTCAGT</td>
<td>For insertion in pENTR™/D-TOPO</td>
</tr>
<tr>
<td>8</td>
<td>C11orf83-R (with STOP)</td>
<td>TCACGGTGACCTCCCGCG</td>
<td>For insertion in pENTR™/D-TOPO</td>
</tr>
<tr>
<td>9</td>
<td>△23C11orf83-F</td>
<td>CACCCTCCTCGTTATCGTGACCCCGG</td>
<td>For insertion in pENTR™/D-TOPO</td>
</tr>
<tr>
<td>10</td>
<td>△23C11orf83-R</td>
<td>TCACGGTGACCTCCCGCG</td>
<td>For insertion in pENTR™/D-TOPO</td>
</tr>
<tr>
<td>11</td>
<td>Helix 3-F</td>
<td>CACCCTGAGGAGACCCAGGAGCAGGGAG</td>
<td>For insertion in pENTR™/D-TOPO</td>
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<tr>
<td>12</td>
<td>Helix3-R</td>
<td>TTATCACCAGTTTCTTCTCCAGGCCAGTTTC</td>
<td>For insertion in pENTR™/D-TOPO</td>
</tr>
<tr>
<td>13</td>
<td>Helix 2-F</td>
<td>CACCCTCCTCGTTATCGTGACCCCGGAGAGCGCGGAAGCAGGAAATGCTAAAGGAGATGCCATAATGA</td>
<td>Anneling and insertion in pENTR™/D-TOPO</td>
</tr>
<tr>
<td>14</td>
<td>Helix 2- R</td>
<td>TCATTATGGCATTCTCCTTTCTACTTCCTGCTTCGCGCGCTCTCCCGGTTACGATAACGAGAGCGCGGATTTGCG</td>
<td>Anneling and insertion in pENTR™/D-TOPO</td>
</tr>
</tbody>
</table>
II. Methods

II.1. Cell lines, DNA transfection and siRNA transfection

HeLa cells were maintained at 37°C with 5% CO₂/95% air in DMEM, low glucose (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies). To establish HeLa cell lines stably down-regulating C11orf83 (sh-1 and sh-2) and sh control cell lines, HeLa cells were transfected with shRNA vectors (pRNAi-H1-puro plasmids, Biosettia) and were selected with 1 µg/ml puromycin according to manufacturer's instructions (Mary et al., 2012). Single colonies were isolated and analyzed for C11orf83 down-regulation by western blotting. The cell lines with the best silencing of C11orf83 were adapted in medium without antibiotic and the stability of the C11orf83 downregulation were regularly checked by western blotting.

DNA transfection was performed with X-tremeGENE transfection reagent (Roche Applied Science) according to the manufacturer's instructions. All the siRNA transfections were performed with Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies) according to the manufacturer's instructions.

II.2. PCR and RT-PCR

RNA of HeLa cells were isolated thanks to the RNeasy kit (Qiagen). This RNA was used for reverse transcription with the Superscript II RNase H Reverse Transcriptase (Invitrogen) and random primers (Promega) according to manufacturer's protocol. The PCR amplification was
performed with an annealing temperature of 58°C, following by one minute of elongation time and 30 cycles of amplification. Primers used for C11orf83: 1 and 2, 3 and 4 for GAPDH.

II.3. Plasmids construction

The C11orf83 primers 1 and 2, described above, were used to amplify a 279 bp DNA fragment containing the C11orf83 coding region from cDNA of HeLa cells: The fragment was cloned into pENTR™/SD/TOPO (Life Technologies) and recombined into the Gateway pcDNA™3.2-DEST vector (Life Technologies) for the expression of C11orf83-V5 fusion protein.

A 88 bp DNA fragment containing the coding sequence for the 23 first aa of C11orf83 was amplified from pcDNA3.2-C11orf83-V5 using primers 5 and 6. After cloning into pENTR™/SD/TOPO, this fragment was recombined into the Gateway pcDNA™-DEST47 vector (Life Technologies) allowing the expression of WT-N23-GFP fusion protein.

For GST-fusion constructs, the C11orf83 full length cDNA was amplified with primers 7 and 8 (GST-C11orf83) and the cDNA of C11orf83 depleted of its N-terminal transmembrane domain (GST-Δ23C11orf83) with primers 9 and 10. The primers 11 and 12 were used to amplify helix 3 sequence from the full length C11orf83 cDNA (GST-Helix 3). The DNA fragment of C11orf83 helix 2 was obtained by annealing of primers 13 and 14 (GST-Helix 2). All these fragments were then cloned in pENTR™/D-TOPO (Life Technologies) and recombined into the Gateway pDEST™15 vector (Life Technologies). The recombinant GST-Helix 2-Helix 3 protein was obtained by site-directed mutagenesis on the pDEST™15 GST-Δ23C11orf83 plasmid using the primers 15 and 16.

The primers 17 and 18 were used to amplify IL6 from a plasmid kindly provided by Giovanni Magistrelli, Novimmune SA (Switzerland). The obtained DNA fragment was inserted in pENTR™/SD/TOPO and recombined into the Gateway pcDNA™3.2-DEST vector for the expression of IL6-V5.
II.4. Site-directed point mutagenesis

Mutated plasmids were obtained by the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, USA). The following primers were used for mutagenesis experiments:

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
<th>Plasmid used</th>
</tr>
</thead>
<tbody>
<tr>
<td>C11orf83-R5AK6A</td>
<td>CAGTCGCAATGGCGGCCGAGGGGCTGGCG</td>
<td>WT-N23-GFP and C11orf83-V5</td>
</tr>
<tr>
<td>C11orf83-R5DK6D</td>
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<td>WT-N23-GFP and C11orf83-V5</td>
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<tr>
<td>C11orf83-L24A</td>
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<td>GST-C11orf83</td>
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<td>C11orf83-K35A</td>
<td>CGGGAGAGCGGCCGACAGAAATGCTAAGGG</td>
<td>GST-C11orf83</td>
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<td>C11orf83-K40A</td>
<td>GAAGCAGGAAATGCTAGCGGAATGCCACTGCAG</td>
<td>GST-C11orf83</td>
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<td>C11orf83-R55A</td>
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<td>GST-C11orf83</td>
</tr>
<tr>
<td>C11orf83-R77A</td>
<td>GAGAAGCTGGCTGGGCAAGAAACTGGGATGGTG</td>
<td>GST-C11orf83</td>
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</table>

II.5. Secretion assay

HeLa cells seeded in 10 cm diameter dishes were transfected with plasmid encoding C11orf83-V5 or IL6–V5. After 48 h, supernatants and cells were harvested. After three washes in PBS, cells were lysed in RIPA buffer for 1 h on ice and centrifuged for 10 min at 18 000 x g to remove cellular debris. Supernatants were concentrated with trichloroacetic acid (10% final) during 30 min on ice and centrifuged for 10 min at 18 000 x g. Pellets were washed several times with PBS to fully remove the trichloroacetic acid, and then resuspended in PBS. Cell lysates (40 μg) and proteins from supernatants (70 μg) were loaded and run on 12% SDS-PAGE gel. After electrophoresis, proteins were transferred onto PVDF membrane to be analyzed by immunoblotting using specific antibodies as indicated. A Ponceau staining on the PVDF membrane was used to control the loading of the different samples.
II.6. Imaging

HeLa cells were grown on coverslips and fixed with cold acetone for 5 min at room temperature (RT) (for anti-C11orf83 antibody only) or with 4% paraformaldehyde for 15 min at RT (for all other antibodies). After three washes in PBS, cells were permeabilized and blocked with 0.25% Triton X-100, 5% bovine serum albumin (BSA) in PBS for 1 h at RT. After blocking, cells were incubated for 2 h at RT with mouse polyclonal anti-C11orf83 (1/100) or mouse monoclonal anti-V5 (1/400) and rabbit monoclonal anti-COX4 (1/400) in PBS containing 5% BSA and 0.25% Triton X-100. After three washes in PBS containing 5% BSA and 0.25% Triton X-100, cells were incubated with secondary antibodies (1/400) for 1 h at RT. The slides were mounted with Mowiol medium containing diamidino-2-phenylindole (DAPI). Images were acquired using a confocal microscope with a 63x oil objective (Carl Zeiss LSM 700, Oberkochen, Germany) and analyzed by Zen software (Carl Zeiss).

II.7. Preparation of the mitochondria-enriched fraction and Na$_2$CO$_3$ and proteinase K assays

HeLa cells were collected in ice-cold mitochondrial buffer (MB, 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.5) with protease inhibitor cocktail (Roche Applied Science). Cells were placed in a precooled glass potter and were broken by 150 strokes using a motor-driven tightly fitting glass/Teflon Potter Elvehjem homogenizer. Homogenates were centrifuged at 600 x g for 10 min at 4°C. The resulting supernatant was further centrifuged at 7000 x g for 10 min at 4°C. The obtained pellet was washed with ice-cold MB, transferred to a 1.5 ml microcentrifuge tube and centrifuged at 7000 x g for 10 min at 4°C. This washed pellet containing mitochondria was resuspended in MB. The protein concentration of this mitochondria-enriched fraction was determined using Bradford method. For the separation of membrane from soluble proteins, 100 µg of proteins from the mitochondria-enriched fraction were treated with 0.1 M sodium carbonate (pH 11) for 20 min on ice. After treatment, the suspension was centrifuged at 100,000 x g for 30 min at 4°C. The pellet (membrane fraction) was resuspended in 50 µl SDS-PAGE loading buffer. The supernatant (soluble proteins) was collected, precipitated using trichloroacetic acid and resuspended in 50 µl of SDS-PAGE loading buffer. An equal volume of each sample was loaded and run on 12% SDS-PAGE gel. After transfer on PVDF membrane (GE Healthcare, Buckinghamshire, UK), an immunoblotting analysis was performed. For
proteinase-K protection assays, 100 µg of proteins from the mitochondria-enriched fraction were pre-incubated with or without 0.5 % saponin for 30 min at 4°C before adding 20 µg proteinase K to obtain a final concentration of 0.25% saponin. In a negative control tube, 5 mM of PMSF was added at the same time that proteinase K. After 30 min RT incubation, proteinase K digestion was stopped with 5 mM PMSF. After loading buffer addition, an equal volume of each sample was loaded and run in a 12% SDS-PAGE gel. Western blot analysis was performed following protein transfer on PVDF membrane.

II.8. Isolation of mitochondria from the mitochondria-enriched fraction

The mitochondria-enriched pellet was resuspended in 1 ml isolation buffer (10 mM Tris-MOPS, 1 mM EGTA/Tris, 250 mM sucrose, pH 7.4) and added on top of a discontinuous sucrose gradient consisting of 19 ml of 1.2 M sucrose, 1 mM EDTA, and 0.1 % BSA in 10 mM HEPES (pH 7.4) over 16 ml of 1.6 M sucrose, 1 mM EDTA, and 0.1 % BSA in 10 mM HEPES (pH 7.4). Samples were placed in a Beckman SW28 rotor and centrifuged at 82,700 x g during 2h20 at 4°C. Mitochondria were recovered at the 1.6 M/1.2 M sucrose buffer interface and resuspended in mitochondria isolation buffer. The protein concentration of these isolated mitochondria was determined using Bradford method.

II.9. Cell growth measurement

For the cell growth measurement, cells were seeded in three 6 cm diameter dishes at 5 x 10⁴ cells/dish. Cells were counted with a Neubauer chamber every day during 3 days. Until six passages after thawing, cells are considered in early passage and in late passage afterwards.

II.10. Apoptosis measurement by flow cytometry

For apoptosis measurement, cells were seeded in 6 cm diameter dishes the day before treatment. Cells were incubated for 8 h with 16 µM actinomycin D in the presence or absence of caspase inhibitor z-VAD-FMK (100 µM). After treatment, apoptosis was detected using annexin V-conjugated APC staining kit (eBioscience, San Diego, USA) according to manufacturer's
instructions. The flow cytometry analyses were performed on a BD Accuri™ C6 Flow Cytometer (BD Bioscience).

II.11. Cellular ATP measurement

HeLa cells were plated at $2 \times 10^4$ cells/well in a 96-well plate the day prior analysis. Cells were then incubated for 1 h in DMEM without glucose supplemented with 4 µM oligomycin or DMSO. ATP levels were determined using the ATP bioluminescence assay kit CLS II according to manufacturer's instructions (Roche Applied Science). Luminescence was monitored in a plate-reader luminometer Fluostar Optima (BMG Labtechnologies, Offenburg, Germany).

II.12. Oxygen consumption measurement

Oxygen consumption was measured with the MitoXpress kit (Luxel Biosciences Ltd, Cork, Ireland) accordingly to the manufacturer's instructions. Briefly, HeLa cells were grown in 96-well plates and incubated for 30 min with 10 µM FCCP, 20 µM rotenone or DMSO in culture medium without phenol red. After the addition of the probe, wells were sealed off with a layer of pre-warmed heavy mineral oil and fluorescence was recorded for 2 h at 30°C on a FLEXstation (Molecular Devices, California, USA) using the time-resolved fluorescence mode.

II.13. Spectrophotometric assays for respiratory complexes

The measurement of the enzymatic activities of the individual complexes of the respiratory chain (RC) was performed by spectrophotometry (UVIKON 922 Spectrophotometer, Kontron, Switzerland). Activities of complexes II, III and IV were measured on total HeLa cell lysates. Activities of complexes I and V were measured on mitochondria-enriched fractions. All samples were frozen and thawed three times before the assays, and assays were performed at RT. To normalize each complex activity, the citrate synthase activity of each sample was determined. A total of 20–60 µg of protein (determined by Bradford method) was used to determine the activity of each complex. Citrate synthase and complexes I, II, III and IV activities were assayed according to Spinazzi and coll. (Spinazzi et al., 2012). The CV activity was measured as described by Kramarova and coll. (Kramarova et al., 2008).
The CI activity was measured at 340 nm using 60 μM ubiquinone as acceptor and 100 μM NADH as donor, in 50 mM potassium phosphate (pH 7.5) buffer containing 3 mg/ml BSA, 300 μM KCN for 2 min. The addition of 10 μM rotenone allowed the quantification of the rotenone-sensitive activity.

The CII activity was performed at 600 nm using 80 μM DCPIP and 20 mM succinate in a medium containing 25 mM potassium phosphate (pH 7.5), 1 mg/ml BSA, 300 μM KCN for 3 min. After 10 min of preincubation at 37°C, the reaction was started by 50 μM of decylubiquinone. The oxidation of succinate was inhibited by 10 mM malonate.

The bc1 complex activity was performed at 550 nm using 75 μM oxidized cytochrome c as acceptor and 100 μM decylubiquinol as donor in a medium containing 25 mM potassium phosphate (pH 7.5), 500 μM KCN, 100 μM EDTA, and 0.025% (v/v) of Tween 20 for 2 min. The addition of 10 μg/μl of antimycin A allowed measuring the specific bc1 complex activity corresponding to the antimycin A-sensitive activity.

The CIV activity was performed at 550 nm using 50 μM reduced cytochrome c in 50 mM potassium phosphate buffer (pH 7.0). The reaction was started by the addition of cell lysate (40 μg proteins). The decrease in absorbance was observed during 3 min. KCN (300 μM) was used to check the specificity of CIV activity.

The CV (F1-ATPase) activity was determined by coupling the reaction to pyruvate kinase and lactate dehydrogenase. The oxidation of NADH was measured at 340 nm using 350 μM of NADH in a medium containing 55 mM Tris (pH 8.0) buffer, 0.05% DDM, 20 mM MgCl₂, 50 mM KCl, 10 mM phosphoenolpyruvate, 30 μM Antimycin A, 10 units of lactate dehydrogenase and 5 units of pyruvate kinase. After incubation at 37°C for 5 min, the reaction was initiated with 2.5 mM ATP and monitored during 3 min. The F1-ATPase-specific activity was determined using 4 μM oligomycin.

The citrate synthase assay was performed at 412 nm to follow the reduction of 100 μM DTNB in the presence of 300 μM acetyl-CoA in 100 mM Tris (pH 8.0) and 0.1% Triton X-100 (v/v) medium. The reaction was started by 500 μM oxalacetic acid and monitored during 3 min.
II.14. Western blotting on whole cell lysates

Cells were lysed with RIPA buffer for 1 h on ice and centrifuged for 10 min at 18,000 x g to remove cellular debris. Cell extract proteins (40 µg) were loaded and run on 12% SDS-PAGE gel. After electrophoresis, proteins were transferred onto PVDF membrane to be analyzed by immunoblotting using specific antibodies as indicated. For western blot quantifications, band intensities obtained by ImageJ Software were normalized to those obtained for sh control cells.

II.15. Blue-Native Polyacrylamide Gel Electrophoresis (BN-PAGE) on isolated mitochondria

Isolated mitochondria were solubilized at 4°C during 30 min, either with 1 g DDM/ g protein to solubilize individual complexes while preserving some interactions between complexes I, III and IV, or with 5 g digitonin/ g protein to maintain SC. After centrifugation (5 min, 18,000 x g, 4 °C), solubilized mitochondrial proteins (40 µg) were loaded and run on a NativePAGE Novex 3-12% Bis-Tris Gel (Life Technologies). For 2D analysis, strips from the first-dimension BN-PAGE were excised from the gel and incubated for 1 h at RT in 1% SDS and 100 mM β-mercaptoethanol, and then subjected in a 12% second-dimension denaturing gel. After electrophoresis, the gels were electroblotted onto PVDF membranes and probed with the indicated antibodies. For western blot quantifications, band intensities obtained by ImageJ Software were normalized to those obtained for sh control cells.

II.16. Co-immunoprecipitation

The mitochondria-enriched fractions were solubilized with DDM (2 g / g protein) and the bc1 complex and CIV were immunoprecipitated using the bc1 complex (ab109800) and CIV (ab109801) immunocapture kits (Abcam) according to manufacturer’s instructions. Proteins were separated by gel electrophoresis on a 12% SDS-PAGE gel, transferred to a PVDF membrane and immunoblotted with anti-UQCRFS1, anti-COX4 and anti-C11orf83 antibodies.

II.17. Thin layer chromatography (TLC)

Lipids were extracted from the mitochondria-enriched fraction thanks to a modified form of the Bligh and Dyer method (Bligh & Dyer, 1959), dissolved in chloroform, and resolved by TLC.
on a silica gel 60 plate (Whatman, Maidstone, UK). For lipid migration, the plate was incubated in solvent containing chloroform: hexane: methanol: acetic acid (50: 30: 10: 5). After a drying step, lipids dots were colored by vapor of iodine crystals. Lipid dots were assigned thanks to lipids standards loaded on the plate at the same time than samples. The quantification of CL and PE spots were obtained by ImageJ Software.

II.18. Phosphate content determination

Phosphate content of spots containing CL and PE on TLC plates was estimated by the method of Rouser and coll. (Rouser et al, 1970).

II.19. Analysis of CL composition by mass spectrometry

CL species present in pellets of cells harvested at confluence were analyzed essentially as described previously (Houtkooper et al, 2009a). The relative abundances of the species in the sample extracts were determined by HPLC-MS using an Ultimate 3000 UPLC system hyphenated to a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Finnigan Corporation, San Jose, CA, USA). The MS was operated in the negative ion electrospray ionization (HESI) mode.

II.20. Recombinant GST protein expression and purification

GST-fusion proteins were expressed in BL21 (DE3)pLys cells (Promega, Madison, USA). The bacteria were grown on LB medium containing 100 µg/mL ampicillin and chloramphenicol and the expression of fusion proteins was induced by 0.5 mM IPTG for 3 h at 30°C. The bacteria were pelleted and resuspended in 100 mM Tris-HCl pH 7.6, 5 mM MgCl₂, 200 mM NaCl, 5 mM DTT, 1% Triton X-100 with protease inhibitors. After sonication, cells debris were pelleted by centrifugation and the supernatants were incubated with glutathione-Sepharose beads. After several washing steps, GST-fusion proteins were eluted with 20 mM glutathione in 100 mM Tris-HCl pH 7.6 and 150 mM NaCl. The homogeneity and integrity of the recombinant proteins were checked by SDS-PAGE followed by Coomassie staining.
II.21. Lipid binding assay

Membrane lipid strips containing dots of 100 nmoles of several lipids (P-6002, Echelon Biosciences Inc., Salt Lake City, USA) were probed with 0.5 ug GST fusion proteins and analysed by immunoblotting according to the manufacturer's instructions.

II.22. Electron microscopy

Cells were fixed with 2% glutaraldehyde in 0.1 M NaPO₄ buffer pH 7.4 for 1 h. After three washing steps with 0.1 M NaPO₄ buffer pH 7.4, cells were dehydrated, embedded in Epoxy resin and processed for electron microscopy as previously described (Foti et al, 1997). Ultrathin sections were finally contrasted with uranyl acetate and lead citrate and observed with a Technai 20 electron microscope (FEI Company, Eindhoven, Netherlands).

II.23. Statistical Analysis

Graphs and data analysis were performed using Graph Pad software (GraphPrism). All values were represented as mean ± SD of values obtained in three independent experiments. Statistical significance (P-value) was assessed by Student’s t test and is indicated by * P<0.05, ** P< 0.01, ***P<0.001 or n.s. (not significant).

II.24. Sequence analysis tools

BLASTP search was performed to find orthologs of C11orf83 (Human) in the UniProtKB database version 15.14. Multiple sequence alignment was performed using T-COFFEE (Notredame et al, 2000) with the sequences of C11orf83 orthologs (UniProtKB sequence identifiers: Q6UW78, Q148G8, Q8K2T4, Q2KP58, E9QEL2, P37267). Secondary structures were predicted with PSIPRED (Jones, 1999) and the multiple sequence alignment was edited using Aline (Bond & Schüttelkopf, 2009). Functional domains and 3D structure similarities were searched using InterProScan (Hunter et al, 2012) and HHPRED (Söding et al, 2005), respectively.
Material and Methods
Appendix
C11orf83, a Mitochondrial Cardiolipin-Binding Protein Involved in
bc1 Complex Assembly and Supercomplex Stabilization

Marjorie Desmurs, Michelangelo Foti, Etienne Raemy, Frédéric Maxime Vaz, Jean-Claude Martinou, Amos Bairoch
Lydie Lane

Mammalian mitochondria may contain up to 1,500 different proteins, and many of them have neither been confidently identified nor characterized. In this study, we demonstrated that C11orf83, which was lacking experimental characterization, is a mitochondrial inner membrane protein facing the intermembrane space. This protein is specifically associated with the bc1 complex of the electron transport chain and involved in the early stages of its assembly by stabilizing the bc1 core complex. C11orf83 displays some overlapping functions with Cbp4p, a yeast bc1 complex assembly factor. Therefore, we suggest that C11orf83, now called UQCC3, is the functional human equivalent of Cbp4p. In addition, C11orf83 depletion in HeLa cells caused abnormal cristae morphology, higher sensitivity to apoptosis, a decreased ATP level due to impaired respiration and subtle, but significant, changes in cardiolipin composition. We showed that C11orf83 binds to cardiolipin by its α-helices 2 and 3 and is involved in the stabilization of bc1 complex-containing supercomplexes, especially the III2/IV supercomplex. We also demonstrated that the OMA1 metalloprotease cleaves C11orf83 in response to mitochondrial depolarization, suggesting a role in the selection of cells with damaged mitochondria for their subsequent elimination by apoptosis, as previously described for OPA1.

Mitochondria are membrane-enclosed organelles composed of several compartments which perform specialized and interconnected functions such as oxidative phosphorylation (OXPHOS), cell death, or carbohydrate and fatty acid metabolisms. OXPHOS, which provides most of the ATP used by the cell, takes place in the inner membrane (IM) and involves five complexes. Redox reactions are carried out by complex I (NADH:ubiquinone oxidoreductase; EC 1.6.5.3), complex II (succinate:ubiquinone oxidoreductase; EC 1.3.5.1), complex III (ubiquinol:ferricytochrome c oxidoreductase; EC 1.10.2.2), and complex IV (cytochrome c oxidoreductase; EC 1.9.3.1). Then, the ATP synthase, complex V (F0F1 ATPase; EC 3.6.3.14), uses the energy released by respiration for ATP generation. The assembly of the OXPHOS complexes requires additional nuclear proteins called assembly factors (1). The physiological importance of these assembly factors is proven by the number of human diseases associated with mutations in genes encoding them (1).

Complex III, also called cytochrome bc1 complex, is a central component of the electron transport chain (ETC). It transfers electrons from coenzyme Q reduced either by complex I through NADH-linked substrates or by complex II through reduced flavin adenine dinucleotide (FADH2)-linked substrates to cytochrome c. The mammalian bc1 complex, which forms as a stable dimer (2), is composed of 11 subunits, among which only MT-CYB is encoded by the mitochondrial genome (3, 4). Most of the work on the bc1 complex assembly has been performed with Saccharomyces cerevisiae and has shown this to be a multistep process involving several subcomplexes and assembly factors (5, 6). Despite the presence of an additional subunit in the mammalian bc1 complex, corresponding to the cleaved precursor of UQCRFS1, the structures of the bc1 complex in yeast and mammals are similar (3, 7).

Whereas 13 bc1 complex assembly factors were identified in yeast, only 5 are currently characterized in mammals: BCSIL (8), TTC19 (9), UQCC1 and UQCC2 (10), and LYRM7 (11). Deleterious mutations in the BCSIL (12) and TTC19 (9) genes were reported to cause GRACILE (growth retardation, amino aciduria, cholestasis, iron overload, lactic acidosis, and early death) syndrome and neurological impairments, respectively, due to a defective bc1 complex assembly.

In the IM, the dimeric bc1 complex (III2) can associate with complex I and/or complex IV to form supercomplexes (SC) (13). The functional role of these SC is still under debate, but recent studies have proposed that they can participate in electron transfer and substrate channeling (14), reduce oxidative damage by decreasing the generation of reactive oxygen species by complex I (15), and confer structural stability on respiratory enzymes (16, 17). Three SC-stabilizing factors were recently identified, COX7A2L in mammals (18, 19) and Rcf1p and Rcf2p in yeast (20, 21), which are orthologs of HIGD1A and HIGD2A, respectively.

In mammals, mitochondria may contain up to 1,500 different proteins (22, 23). About 900 of them have been identified and characterized, but the others lack experimental validation (24, 25). We can speculate that several assembly or stabilizing factors of
OXPHOS complexes and SC are still unidentified. In this study, we determined that C11orf83 is a mitochondrial protein, targeted and anchored in the IM by its N-terminal section. We showed that C11orf83 can bind to the mitochondrial phospholipids cardiolipin (CL) and phosphatidic acid (PA) independently of this N-terminal membrane-anchoring region. C11orf83-deficient cells displayed abnormal mitochondria with an impaired OXPHOS due to a bc1 complex assembly deficiency at early stages and to a decrease of the amounts of SC, especially the III/IV SC. Moreover, we showed that C11orf83 is a target of OMA1, a mitochondrial metalloproteinase, upon mitochondrial depolarization. Taken together, our results demonstrate that C11orf83 is a new assembly factor of the bc1 complex and also a stabilizing factor of the III/IV SC and thus is required for proper mitochondrial morphology and function.

**MATERIALS AND METHODS**

**Reagents and antibodies.** Nigericin, E-64-D, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), oligomycin A, actinomycin D, valinomycin, pepstatin A, and z-VAD-FMK were from Enzo Life Sciences (Lausen, Switzerland). Digitonin was from EMD Millipore (Darmstadt, Germany). Proteinase K was provided by Macherey-Nagel (Düren, Germany). Saponin, n-dodecyl-b-maltoside (DDM), carbonyl cyanide m-chlorophenylhydrazone (CCCP), isoospryl-b-thiolactolate (IPTG), glutathione, glutathione-Phaphorose beads, rotenone, dimethyl sulfoxide (DMSO), N-tosyl-l-phenylalanyl chloromethyl ketone (TPCK), phenylmethylsulfonyl fluoride (PMSF), 3,4-dichloroisocoumarin (DCI), 1,10-phenanthroline (O-PHE), deelyubiquinol, cytochrome c, 2,6-dicholorophenolden phospholium salts (DCPIP), succinic acid, potassium cyanide (KCN), oxaloacetic acid, 5'-dithiothreitol (2-nitrobenzoic acid) (DTNB), malonic acid, ubiquinone, lactate dehydrogenase, pyruvate kinase, phosphonolipynurycate, and antinucie A were from Sigma-Aldrich (St. Louis, MO).

Mouse polyclonal anti-C11orf83 polyonal antibody (PAb) was from Pirmm (Milan, Italy). Mouse monochoral anti-OPA1 antibody (d16606) was from BD Biosciences (NJ). Sheep polyclonal anti-cytochrome c antibody (ab89679) was from Abcam (Cambridge, United Kingdom). Rabbit polyclonal antibodies obtained from commercial sources were anti-FISI (sc-9800), and anti-SIRT3 (sc-99143) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-COX4 from Cell Signaling Technologies (Danvers, MA), anti-OMAI (NB81-56790) from Novus Biological (Littleton, CO), anti-b-catenin (C2026) from Sigma-Aldrich, and anti-DIABLO (ADI-905-244) from Enzo Life Sciences. All the other rabbit polyclonal antibodies were kindly provided by the Human Protein Atlas team (26); anti-ATP5 (HPA010520), anti-C11orf83 (HPA046851), anti-CYCI (HPA001247), anti-glycereraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (HPA040067), anti-NDUFB6 (HPA040401), anti-NDUFA2 (HPA042757), anti-SDHB (HPA002868), anti-TIMM44 (HPA043052), anti-UQCRC1 (HPA003606), anti-UQCRC1 (HPA002815), anti-UQCRC1 (HPA004863), and anti-UQCRC2 (HPA006993). The mouse antibodies against tags were polyclonal anti-V5 (MC2892GA; Abd Serotec, Raleigh, NC), monoclonal anti-glutathione S-transferase (anti-GST) (ab92; Abcam), and monoclonal anti-green fluorescent protein (anti-GFP) (118146000; Roche Applied Science, Penzberg, Germany). Secondary antibodies for Western blotting were rabbit anti-sheep IgG (sc-2770; Santa Cruz Biotechnology) and goat anti-mouse and anti-rabbit IgG coupled to horseradish peroxidase (62-6520 and G2-12134, respectively; Life Technologies, Carlsbad, CA). For immunofluorescence, secondary antibodies were goat anti-mouse antibody conjugated with Alexa 488 and anti-rabbit antibody conjugated with rhodamine (Life Technologies).

**Cell lines, DNA transfection, and siRNA transfection.** HeLa cells were maintained at 37°C with 5% CO2—95% air in Dulbecco’s modified Eagle medium (DMEM), low glucose (Life Technologies), supplemented with 10% fetal bovine serum (Life Technologies). HeLa cells stable downregulating C11orf83 (sh-1 and sh-2) and sh control (sh CTL) cell lines were established as described previously (27). DNA transfection was performed with X-tremeGENE transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. All the small interfering RNA (siRNA) transfections were performed with Lipofectamine RNAiMAX transfection reagent (Life Technologies) according to the manufacturer’s instructions.

**cDNA cloning, plasmid construction, site-directed point mutagenesis, shRNA sequence, and siRNA.** The following primers were used to amplify a 279-bp DNA fragment containing the C11orf83 coding region from cDNA of HeLa cells: 5’-CGGATGACTCCCTCCGGCCGGC-3’ and 5’-CGGTGACCTTCGCCGCGC-3’. The fragment was cloned into pENTR/SD-D-TOPO (Life Technologies) and recombined into the Gateway pcDNA3.2-DEST vector (Life Technologies) for the expression of C11orf83-V5 fusion protein. An 88-bp DNA fragment containing the coding sequence for the first 23 amino acids (aa) of C11orf83 was amplified from cDNA of C11orf83 and used 5’-ACCA TGATTTCTTTCGGGAAAATAGCTGATCTCC-3’ and 5’-CCGTGACCTTCGCCGCGC-3’ as primers. After being cloned into pENTR/SD-D-TOPO, this fragment was recombined into the Gateway pcDNA3.2-DEST vector (Life Technologies), allowing the expression of WT-N23-GFP fusion protein. Mutated plasmids were obtained by the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The following primers were used for mutagenesis experiments: RSAK5a (5’-CAGTGGCAGATCCCAGGGGCG-3’) and RSDK5d (5’-CCACATATTGACGATCATCGTTCGCTAAGAATCATTG-3’). For GST fusion constructs, the primers used to amplify the C11orf83 full-length cDNA and the cDNA of C11orf83 depleted of its N-terminal transmembrane domain (Δ23C11orf83) were either 5’-ACCGATTTCCCTCGGAGGATTAGTCGATCAGT-3’ or 5’-ACCCCTCTCTGTATGCTG-3’. For GST fusion constructs, the primers used to amplify the C11orf83 full-length cDNA and the cDNA of C11orf83 depleted of its N-terminal transmembrane domain (Δ23C11orf83) were either 5’-ACCGATTTCCCTCGGAGGATTAGTCGATCAGT-3’ or 5’-ACCCCTCTCTGTATGCTG-3’. For GST fusion constructs, the primers used to amplify the C11orf83 full-length cDNA and the cDNA of C11orf83 depleted of its N-terminal transmembrane domain (Δ23C11orf83) were either 5’-ACCGATTTCCCTCGGAGGATTAGTCGATCAGT-3’ or 5’-ACCCCTCTCTGTATGCTG-3’. For GST fusion constructs, the primers used to amplify the C11orf83 full-length cDNA and the cDNA of C11orf83 depleted of its N-terminal transmembrane domain (Δ23C11orf83) were either 5’-ACCGATTTCCCTCGGAGGATTAGTCGATCAGT-3’ or 5’-ACCCCTCTCTGTATGCTG-3’. For GST fusion constructs, the primers used to amplify the C11orf83 full-length cDNA and the cDNA of C11orf83 depleted of its N-terminal transmembrane domain (Δ23C11orf83) were either 5’-ACCGATTTCCCTCGGAGGATTAGTCGATCAGT-3’ or 5’-ACCCCTCTCTGTATGCTG-3’. For GST fusion constructs, the primers used to amplify the C11orf83 full-length cDNA and the cDNA of C11orf83 depleted of its N-terminal transmembrane domain (Δ23C11orf83) were either 5’-ACCGATTTCCCTCGGAGGATTAGTCGATCAGT-3’ or 5’-ACCCCTCTCTGTATGCTG-3’. For GST fusion constructs, the primers used to amplify the C11orf83 full-length cDNA and the cDNA of C11orf83 depleted of its N-terminal transmembrane domain (Δ23C11orf83) were either 5’-ACCGATTTCCCTCGGAGGATTAGTCGATCAGT-3’ or 5’-ACCCCTCTCTGTATGCTG-3’. For GST fusion constructs, the primers used to amplify the C11orf83 full-length cDNA and the cDNA of C11orf83 depleted of its N-terminal transmembrane domain (Δ23C11orf83) were either 5’-ACCGATTTCCCTCGGAGGATTAGTCGATCAGT-3’ or 5’-ACCCCTCTCTGTATGCTG-3’.
Preparation of the mitochondrion-enriched fraction and Na$_2$CO$_3$ and proteinase K assays. HeLa cells were collected in ice-cold mitochondrial buffer (MB; 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA (pH 7.5)) with protease inhibitor cocktail (Roche Applied Science). Cells were plated in a precooled glass Potter-Elvehjem homogenizer and were broken by 150 strokes using a motor-driven tightly fitting glass/Teflon Potter-Elvehjem homogenizer. Homogenates were centrifuged at 600 × g for 10 min at 4°C. The resulting supernatant was further centrifuged at 7,000 × g for 10 min at 4°C. The obtained pellet was washed with ice-cold MB, transferred to a 1.5-ml microcentrifuge tube, and centrifuged at 7,000 × g for 10 min at 4°C. This washed pellet containing mitochondria was resuspended in MB. The protein concentration of this mitochondrion-enriched fraction was determined using the Bradford method. For the separation of membrane from soluble proteins, 100 μg of proteins from the mitochondrion-enriched fraction were treated with 0.1 M sodium carbonate (pH 11) for 20 min on ice. After treatment, the suspension was centrifuged at 100,000 × g for 30 min at 4°C. The pellet (membrane fraction) was resuspended in 50 μl of SDS-PAGE loading buffer. The supernatant (soluble proteins) was collected, precipitated using trichloroacetic acid, and resuspended in 50 μl of SDS-PAGE loading buffer. An equal volume of each sample was loaded and run on a 12% SDS-PAGE gel. After transfer to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Buckinghamshire, United Kingdom), an immunoblotting analysis was performed. For proteinase K protection assays, 100 μg of proteins from the mitochondrion-enriched fraction was preincubated with and without 0.5% saponin for 30 min at 4°C before addition of 20 μg of proteinase K to obtain a final concentration of 0.25% saponin. In a negative-control tube, 5 mM PMSF was added at the same time as proteinase K. After 30 min of RT incubation, proteinase K digestion was stopped with 5 mM PMSF. After loading buffer addition, an equal volume of each sample was loaded and run in a 12% SDS-PAGE gel. Western blot analysis was performed following protein transfer to a PVDF membrane.

Isolation of mitochondria from the mitochondrion-enriched fraction. The mitochondrion-enriched pellet was resuspended in 1 ml of isolation buffer (10 mM Tris-morpholinepropanesulfonic acid [MOPS], 1 mM EDTA–Tris, 250 mM sucrose (pH 7.4)) and added on top of a discontinuous sucrose gradient consisting of 19 ml of 1.2 M sucrose, 1 mM EDTA, and 0.1% BSA in 10 mM HEPES (pH 7.4) over 14 ml of 1.6 M sucrose, 1 mM EDTA, and 0.1% BSA in 10 mM HEPES (pH 7.4). Samples were placed in a Beckman SW28 rotor and centrifuged at 82,700 × g for 2 h 20 min at 4°C. Mitochondria were recovered at the 1.6 M/1.2 M sucrose buffer interface and resuspended in mitochondrial isolation buffer. The protein concentration of these isolated mitochondria was determined using the Bradford method.

Cell growth measurement. For the cell growth measurement, cells were seeded in three 6-cm-diameter dishes at 5 × 10$^5$ cells/dish. Cells were counted with a Neubauer chamber every day for 3 days. Until six passages after thawing, cells are considered in early passage; they are considered in late passage afterwards.

Apoptosis measurement by flow cytometry. For apoptosis measurement, cells were seeded in 6-cm-diameter dishes the day before treatment. Cells were incubated for 8 h with 16 μM actinomycin D in the presence or absence of caspase inhibitor z-VAD-FMK (100 μM). After treatment, apoptosis was detected using an aliphophycocyanin (APC)-conjugated annexin V staining kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. The flow cytometry analyses were performed on a BD Accuri C6 flow cytometer (BD Bioscience).

Cellular ATP measurement. HeLa cells were plated at 2 × 10$^4$ cells/well in a 96-well plate the day prior to analysis. Cells were then incubated for 1 h in DMEM without glucose supplemented with 4 μM oligomycin or DMSO. ATP levels were determined using the ATP bioluminescence assay kit CLS II according to the manufacturer's instructions (Roche Applied Science). Luminescence was monitored in a Fluostar Optima plate reader luminometer (BMG Lab Technologies, Offenburg, Germany).

Oxygen consumption measurement. Oxygen consumption was measured with the MitoXpress kit (Luxel Biosciences Ltd., Cork, Ireland) accordingly to the manufacturer’s instructions. Briefly, HeLa cells were grown in 96-well plates and incubated for 30 min with 10 μM FCCP and 20 μM rotenone or DMSO in culture medium without phenol red. After the addition of the probe, wells were sealed off with a layer of prewarmed heavy mineral oil and fluorescence was recorded for 2 h at 30°C on a FLEXstation ( Molecular Devices, CA) using the time-resolved fluorescence mode.

Spectrophotometric assays for respiratory complexes. The measurement of the enzymatic activities of the individual complexes of the respiratory chain (RC) was performed by spectrophotometry (UVikon 922 spectrophotometer; Kontron, Switzerland). Activities of complexes II, III, and IV were measured on total HeLa cell lysates. Activities of complexes I and V were measured on mitochondrion-enriched fractions. All samples were frozen and thawed three times before the assays, and assays were performed at RT. To normalize each complex activity, the citrate synthase activity of each sample was determined. A total of 20 to 60 μg of protein (determined by the Bradford method) was used to determine the activity of each complex. Citrate synthase and complex I, II, III, and IV activities were assayed according to the method of Spinazzi and colleagues (28). Complex V activity was measured as described by Kramarova and colleagues (29).

Complex I activity was measured at 340 nm using 60 μM ubiquinone as an acceptor and 100 μM NADH as a donor, in 50 mM potassium phosphate (pH 7.5) buffer containing 3 mg/ml of BSA and 300 μM KCN for 2 min. The addition of 10 μM rotenone allowed the quantification of the rotenone-sensitive activity.

Complex II activity was determined at 600 nm using 80 μM DCPIP and 20 mM succinate in a medium containing 25 mM potassium phosphate (pH 7.5), 1 mg/ml of BSA, and 300 μM KCN for 3 min. After 10 min of preincubation at 37°C, the reaction was started by 50 μM decylubiquinone. The oxidation of succinate was inhibited by 10 mM malonate.

Complex III activity was determined at 530 nm using 75 μM oxidized cytochrome c as an acceptor and 100 μM decylubiquinol as a donor in a medium containing 25 mM potassium phosphate (pH 7.5), 500 μM KCN, 100 μM EDTA, and 0.025% (vol/vol) Tween 20 for 2 min. The addition of 10 μg/ml of antimycin A allowed measurement of the specific complex III activity corresponding to the antimycin A-sensitive activity.

Complex IV activity was determined at 550 nm using 50 μM reduced cytochrome c in 50 mM potassium phosphate buffer (pH 7.0). The reaction was started by the addition of cell lysate (40 μg of proteins). The decrease in absorbance was observed for 3 min. KCN (300 μM) was used to check the specificity of complex IV activity.

Complex V (F$_1$F$_0$ ATPase) activity was determined by coupling the reaction to pyruvate kinase and lactate dehydrogenase. The oxidation of NADH was measured at 340 nm using 350 μM NADH in a medium containing 55 mM Tris (pH 8.0) buffer, 0.05% DDM, 20 mM MgCl$_2$, 50 mM KCl, 10 mM phosphoenolpyruvate, 30 μM antimycin A, 10 μM lactate dehydrogenase, and 5 μU of pyruvate kinase. After incubation at 37°C for 5 min, the reaction was initiated with 2.5 mM ATP and monitored for 3 min. The F$_1$F$_0$ ATPase-specific activity was determined using 4 μM oligomycin.

The citrate synthase assay was performed at 412 nm to follow the reduction of 100 μM DTNB in the presence of 300 μM acetyl coenzyme A (acetyl-CoA) in 100 mM Tris (pH 8.0) and 0.1% (vol/vol) Triton X-100 medium. The reaction was started by 500 μM oxalacetic acid and monitored for 3 min.

Western blotting on whole-cell lysates. Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer for 1 h on ice and centrifuged for 10 min at 18,000 × g to remove cellular debris. Cell extract proteins (40 μg) were loaded and run on a 12% SDS-PAGE gel. After electrophoresis, proteins were transferred to a PVDF membrane to be analyzed by immunoblotting using specific antibodies as indicated below. For Western blotting...
quantifications, band intensities obtained by ImageJ software were normal- zed to those obtained for sh control cells.

**BN PAGE on isolated mitochondria.** Isolated mitochondria were solubilized at 4°C during 30 min, either with 1 g of DDM/ml of protein to solubilize individual complexes while preserving some interactions be- tween complexes I, III, and IV or with 5 g of dixitong/m of protein to maintain SC. After centrifugation (5 min, 18,000 × g, and 4°C), solubi- lized mitochondrial proteins (40 µg) were loaded and run on a native PAGE Novex 3 to 12% bis-Tris gel (Life Technologies). For two-dimen- sional (2D) analysis, strips from the first-dimension blue native polyacryl- amide gel electrophoresis (BN PAGE) were excised from the gel, incu- bated for 1 h at RT in 1% SDS and 100 mM β-mercaptoethanol, and then subjected to a 12% second-dimension denaturing gel electrophoresis (BN/SDS-PAGE). After electrophoresis, the gels were electroblotted onto PVDF membranes and probed with the desired antibodies. For Western blot quantifications, band intensities obtained by ImageJ software were normalized to those obtained for sh control cells.

**TLC.** Lipids were extracted from the mitochondrion-enriched fraction thanks to a modified form of the method of Bligh and Dyer (30), dissolved in chloroform, and resolved by thin-layer chromatography (TLC) on a silica gel 60 plate (Whatman, Maidstone, United Kingdom). For lipid migration, the plate was incubated in solvent containing chloro- form, hexane, methanol, and acetic acid (50:30:10:5). After a drying step, lipids dots were colored by vapor of iodine crystals. Lipid dots were as- signed on the basis of lipid standards loaded on the plate at the same time as samples. The quantification of CL and phosphatidylethanolamine (PE) spots were obtained by ImageJ software.

**Phosphate content determination.** Phosphate content of spots contain- ing CL and PE on TLC plates was estimated by the method of Rouser and colleagues (31).

**Analysis of CL composition by MS.** CL species present in pellets of cells harvested at confluence were analyzed essentially as described previ- ously (32). The relative abundances of the species in the sample extracts were determined by high-performance liquid chromatography (HPLC)-mass spectrometry (MS) using an Ultimate 3000 ultraperformance liquid chromatography (UPLC) system in conjunction with a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer (Thermo Finnigan Cor- poration, San Jose, CA). The MS was operated in the negative-ion heated electrospray ionization (HESI) mode.

**Recombinant GST protein expression and purification.** GST fusion proteins were expressed in BL21(DE3)pLYs cells (Promega, Madison, WI). The bacteria were grown on LB medium containing 100 µg/ml of ampicillin, 100 µg/ml of chloramphenicol, and the expression of fusion proteins was induced by 0.5 mM IPTG for 3 h at 30°C. The bacteria were pelleted and resuspended in 100 mM Tris-HCl (pH 7.6), 5 mM MgCl2, 200 mM NaCl, 5 mM dithiothreitol (DTT), and 1% Triton X-100 with protease inhibitors. After sonication, cells debris were pelleted by centrifugation and the supernatants were incubated with glutathione-Sepharose beads. After several washing steps, GST fusion proteins were eluted with 20 µM glutathione in 100 mM Tris-HCl (pH 7.6) and 150 mM NaCl. The homogeneity and integrity of the recombinant proteins were checked by SDS- PAGE followed by Coomassie staining.

**Lipid binding assay.** Membrane lipid strips containing dots of 100 nmol of several lipids (P-6002; Echelon Biosciences Inc., Salt Lake City, UT) were probed with 0.5 µg of GST fusion proteins and analyzed by immunoblotting according to the manufacturer’s instructions.

**Electron microscopy.** Cells were fixed with 2% glutaraldehyde in 0.1 M NaPO4 buffer (pH 7.4) for 1 h. After three washing steps with 0.1 M NaPO4 buffer (pH 7.4), cells were dehydrated, embedded in epoxy resin, and processed for electron microscopy as previously described (33). Ultrathin sections were finally contrasted with uranyl acetate and lead citrate and observed with a Tecnai 20 electron microscope (FEI Com- pany, Eindhoven, The Netherlands).

**Coimmunoprecipitation.** The mitochondrion-enriched fractions were solubilized with DDM (2 g/g of protein), and the bc1 complex and complex IV were immunoprecipitated using the bc1 complex (ab199800) and complex IV (ab109801) immunocapture kits (Abcam) according to the manufacturer’s instructions. Proteins were separated by gel electrophoresis on a 12% SDS-PAGE gel, transferred to a PVDF membrane, and immunoblotted with anti-UQCRFS1, anti-COX4, and anti-C11orf83 an- tibodies.

**Statistical analysis.** Graphing and data analysis were performed using GraphPad software (GraphPrism). All values are represented as means ± standard deviations (SDs) of values obtained in three independent exper- iments. Statistical significance (P value) was assessed by Student’s t test.

**Sequence analysis tools.** BLASTP search was performed to find or- thologs of C11orf83 (human) in the UniProtKB database version 15.14. Multiple-sequence alignment was performed using T-COFFEE (34) with the sequences of C11orf83 orthologs (UniProtKB [www.uniprot.org]), se- quence identifiers: Q6UW78, Q148G8, Q8K2T4, and Q2KP58. Second- ary structures were predicted with PSIPRED (35), and the multiple-se- quence alignment was edited using Aline (36). Functional domains and three-dimensional structure similarities were searched using InterPro (37) and HHPPRED (38), respectively.

**RESULTS**

**C11orf83 is an integral mitochondrial inner membrane protein facing the intermembrane space (IMS).** C11orf83 is a protein of 10.1 kDa (93 amino acids [aa]) which is highly conserved in Meta- zona. A similarity of 50% between human and Xenopus laevis pro- tein sequences was determined using the sequence alignment pre- sented in Fig. 1A and the BLOSUM64 matrix. In addition, secondary-structure prediction (PSIPRED) indicated the pres- ence of conserved secondary structures (Fig. 1A). However, no obvious functional domain was found (InterPro), and no similar- ity to any protein with a resolved three-dimensional structure could be retrieved by HHPPRED. Bioinformatic analysis, as well as a large-scale experimental study, originally predicted that C11orf83 was a secreted protein due to the presence of a potential signal peptide of 23 aa in length (Fig. 1A) (39, 40). However, C11orf83 was recently identified among the membrane proteins in a proteomic study of crude mitochondrial extracts from HeLa S3 cells (41). Therefore, we decided to analyze the localization of C11orf83 in HeLa cells by confocal microscopy and subcellular frac- tionation. As shown in Fig. 1B, C11orf83 colocalized with COX4, a mitochondrial protein. After Na2CO3 treatment of the mitochondri- on-enriched fraction, membrane proteins, like TOMM20, were found in the pellet and soluble proteins, like DIABLO, were retained in the supernatant. C11orf83 was found in the membrane protein pellet (Fig. 1C), suggesting that it is a mitochondrial integral mem- brane protein, in agreement with the results of the proteomic study (41).

To determine C11orf83 membrane topology, we performed a proteinase K protection assay. Mitochondrion-enriched fractions from HeLa cells were treated with proteinase K in the presence or absence of saponin, which is used to permeabilize the mitochon- drial outer membrane (OM). In contrast to the IM protein TIMM44, which is oriented toward the mitochondrial matrix, C11orf83 was digested by proteinase K in the presence of saponin, as was the case for DIABLO, a soluble protein from the IMS, and OPA1, a mitochondrial IM protein facing the IMS (Fig. 1D, lane 5). Taken together, these data indicate that C11orf83 is a mito- chondrial IM protein facing the IMS. We performed the same assay using HeLa cells overexpressing the C-terminally V5-tagged form of C11orf83 (C11orf83-V5) and observed similar localization and membrane topology (see Fig. SA1A to C in the supple- mental material).
FIG 1 Subcellular localization of C11orf83 and identification of the membrane-anchoring sequence. (A) Sequence alignment of human C11orf83 with its orthologs. UniProtKB accession numbers of sequences are Q6UW78 (human), Q148G8 (bovine), Q8K2T4 (mouse), and Q2KP58 (Xenopus laevis). Predicted α-helices (cylinders) and β-strand (arrow) are displayed above the alignment. The conserved RK motif is highlighted by a red box. (B) Immunofluorescence analysis of C11orf83 localization in HeLa cells by confocal microscopy. Endogenous C11orf83 (anti-C11orf83 PAb [green]) was colocalized with COX4 (anti-COX4 PAb [red]). A merged color image is shown. (Scale bars: 10 μm in the large image and 2 μm in the small images.) (C) The mitochondrion-enriched fraction from HeLa cells was subjected to Na₂CO₃ extraction and analyzed by immunoblotting. C11orf83 and TOMM20 were retained in the insoluble membrane pellet (P), whereas the soluble protein DIABLO was released into the supernatant (S). I, input. (D) Mitochondrion-enriched fractions from HeLa cells were digested by proteinase K in the absence (lane 2) or presence (lane 5) of saponin and analyzed by immunoblotting. C11orf83 was only digested by proteinase K in the presence of saponin (lane 5), indicating that C11orf83 is in the IM and faces the IMS. M, matrix. (E) The NH₂-terminal part of C11orf83 (23 aa) was fused to GFP for expression in HeLa cells (WT-N23-GFP). Introduced mutations are shown in red in the corresponding sequences. (F) Immunofluorescence analysis of WT-N23-GFP localization in HeLa cells by confocal microscopy. WT-N23-GFP fusion protein was colocalized with COX4 (anti-COX4 PAb [red]). A merged color image is shown. (Scale bars: 10 μm in the large image and 2 μm in the small images.) (G) The mitochondrion-enriched fraction from HeLa cells transfected with WT-N23-GFP was subjected to Na₂CO₃ extraction and analyzed by immunoblotting. WT-N23-GFP (labeled with anti-GFP) and COX4 were retained in the insoluble membrane pellet (P). The minor band detected by the anti-GFP antibody may reflect protein degradation. (H) Immunofluorescence analysis of HeLa cells transfected with WT-N23-GFP or its mutated forms by confocal microscopy. The WT-N23-GFP fusion protein was colocalized with COX4 (anti-COX4 PAb [red]), whereas R5AK6A- and R5DK6D-N23-GFP fusion proteins were mislocalized in the cytosol. Merged color images are shown. (Scale bars: 10 μm in the large images and 2 μm in the small images, which are enlargements of the dashed boxes.)
Therefore, we hypothesized that the hydrophobic N-terminal part of C11orf83, predicted to be a secretory signal peptide (39, 40), would instead serve as a mitochondrial targeting signal and a membrane-anchoring region. To test this hypothesis, we expressed a modified form of GFP with this peptide fused at its N terminus (WT-N23-GFP) in HeLa cells. By confocal microscopy, we observed a clear colocalization of the mitochondrial COX4 signal with the WT-N23-GFP signal (Fig. 1E and F). The mitochondrion-enriched fraction from HeLa cells expressing WT-N23-GFP was then subjected to Na\textsubscript{2}CO\textsubscript{3} treatment. WT-N23-GFP was found in the pellet corresponding to the membrane protein fraction (Fig. 1G). These data suggest that this N-terminal sequence is sufficient to target and anchor C11orf83 to the mitochondrial IM. We identified a conserved RK motif in this N-terminal region (Fig. 1A, red box). We replaced these 2 residues either with alanine or with aspartic acid (Fig. 1E) and analyzed the localization of these mutated N23-GFP forms in HeLa cells. Mutations in the RK motif abrogated N23-GFP targeting to the mitochondria and induced a cytoplasmic localization (Fig. 1H). We observed similar results with RK-mutated C11orf83-V5 (see Fig. SA1D and E in the supplemental material), which confirmed the key role of these two conserved and positively charged amino acids for the correct mitochondrial localization of C11orf83.

C11orf83 downregulation impairs mitochondrial functions. To investigate the biological function of C11orf83 at the cellular level, we engineered HeLa cell lines by stably downregulating C11orf83 using shRNA technology. Two cell lines, called sh-1 and sh-2, were chosen due to their absence of detectable C11orf83 protein expression (Fig. 2A). We noticed that C11orf83 downregulation was more stable in sh-2 cells than in sh-1 cells (data not shown). On freshly thawed cell lines (less than six passages), we observed similar growth curves for cells downregulating C11orf83 and controls (Fig. 2B). However, we noticed that after six passages, C11orf83-deficient cells showed a significantly reduced cell growth. At the molecular level, C11orf83 downregulation increased the susceptibility of HeLa cells to actinomycin D-induced apoptosis (Fig. 2C). Moreover, C11orf83 downregulation impaired ATP production (Fig. 2D) and basal respiration (Fig. 2E).
TABLE 1 Enzymatic activities of mitochondrial RC complexes in WT, sh control, and C11orf83-deficient cells (sh-1 and sh-2)∗

<table>
<thead>
<tr>
<th>Complex</th>
<th>HeLa WT</th>
<th>sh-1</th>
<th>sh-2</th>
<th>sh CTL</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>26.4 ± 1.6</td>
<td>20.1 ± 1.0*</td>
<td>17.2 ± 2.9**</td>
<td>31.3 ± 1.9</td>
</tr>
<tr>
<td>II</td>
<td>7.8 ± 3.5</td>
<td>4.8 ± 1.5*</td>
<td>4.0 ± 1.0**</td>
<td>8.7 ± 1.8</td>
</tr>
<tr>
<td>III</td>
<td>94.0 ± 13.9</td>
<td>62.5 ± 23.4*</td>
<td>70.9 ± 23.7*</td>
<td>128.2 ± 46.0</td>
</tr>
<tr>
<td>IV</td>
<td>100.1 ± 13.0</td>
<td>61.2 ± 8.8*</td>
<td>79.9 ± 3.5*</td>
<td>121.7 ± 21.3</td>
</tr>
<tr>
<td>V</td>
<td>25.8 ± 6.3</td>
<td>13.4 ± 5.6*</td>
<td>10.6 ± 0.3*</td>
<td>28.3 ± 10.4</td>
</tr>
</tbody>
</table>

∗Values are normalized to citrate synthase activities and reported as means ± SDs (n = 3). *, P < 0.05; **, P < 0.01 (compared to sh CTL values).

growth compared to that of controls (see Fig. SA2 in the supplemental material). These data suggest that the C11orf83 loss has a potential impact on resistance to in vitro cellular aging.

Since C11orf83 was shown to be mitochondrial and several studies proposed a connection between aging and mitochondria dysfunctions (42–46), a deeper analysis of the impact of C11orf83 depletion on mitochondrial physiology was performed. To comply with the instructions of the American Type Culture Collection (ATCC) (47), which recommend the use of cell lines within five passages to ensure reliable and reproducible results, all the following experiments were performed with freshly thawed cells. As mitochondria play a key role in apoptosis and ATP production, we first monitored the sensitivities of these cell lines to actinomycin D-induced apoptosis, as well as their ATP levels. Upon actinomycin D treatment, we observed a significant increase in annexin V-positive cells among C11orf83-deficient cells by flow cytometry analysis (Fig. 2C). The assay specificity was confirmed by the inhibition of annexin V staining in the presence of z-VAD-FMK, a pan-caspase inhibitor. These results showed that the knockdown of C11orf83 induced a higher sensitivity to this apoptotic stimulus. Next, we measured the ATP level of each cell line using a luciferase reporter assay. The C11orf83-deficient cell lines sh-1 and sh-2 showed 40% and 30% reductions in ATP levels compared to controls, respectively (Fig. 2D). Since the cellular ATP is mainly provided by mitochondria via the ETC, we wondered if the observed decrease in cellular ATP could be due to an impaired respiration. Using a cell-based assay, we showed that the loss of C11orf83 induced a significant decrease of basal respiration (Fig. 2E). Taken together, these results suggest that C11orf83 is important for proper mitochondrial physiology.

C11orf83 depletion induces defects in respiratory SC. As the loss of C11orf83 induced a decrease in basal respiration, we investigated its potential effects on individual RC complexes. In Table 1, we summarize the activities of the five RC complexes in knockdown and control cells measured by spectrophotometry analysis. All complexes in C11orf83 knockdown cells demonstrated a significantly lower activity than in controls.

Then, we examined the effects of C11orf83 downregulation on the steady-state levels of proteins that form ETC complexes. Immunoblotting on whole-cell lysates, under denaturing conditions, showed that the loss of C11orf83 did not affect the amount of representative subunits for each complex (NDUFB2 for complex I, SDHB for complex II, UQCRHC1 for bc1 complex, COX4 for complex IV, and ATP5B for complex V) (Fig. 3A).

To determine whether the assembly of ETC complexes and SC was affected in C11orf83-depleted cells, we performed blue native (BN) gel electrophoresis followed by immunoblotting on isolated mitochondria solubilized by DDM. Under this condition, complexes II and V were extracted as individual complexes (visualized with antibodies against SDHB for complex II and ATP5B for complex V) (Fig. 3B). Complexes I and IV and the bc1 complex dimer were also extracted as individual complexes and additionally found as III1/IV2, III1/III2, and III1/IV5 SC (visualized with antibodies against NDUF86 for complex I, UQRC1 for bc1 complex, and COX4 for complex IV) (Fig. 3C). C11orf83 depletion caused no apparent change in the detectable amounts of complexes I, II, III2, IV, and V and of the I/III1 SC (Fig. 3B and C). In contrast, we observed a significant reduction in the amounts of the III1/IV and III1/IV5 SC, with a more pronounced effect for the III1/IV SC (Fig. 3C, D, and E).

C11orf83 interacts with the bc1 complex and is involved in the early stages of bc1 assembly. To determine the cause of the reduced amounts of the I/III1/IV and III1/IV5 SC, we solubilized mitochondria using digitonin, which better preserves SC integrity than DDM, and performed two-dimensional (2D) BN/SDS-PAGE followed by immunoblotting. When this protocol was applied to control cells, complex I (visualized with antibody against NDUFV2) was found uniquely in the I/III1/IV SC, whereas complexes IV and III1 (visualized with antibodies against COX4 and UQRC1, respectively) were found both as individual complexes and in the III1/IV and I/III1/IV5 SC, as expected from the literature (16, 48) (Fig. 4A). In mitochondria from sh-2 cells solubilized with digitonin, UQRC1 was predominantly found in the I/III1/IV5 SC, and we observed a significant reduction in the amounts of complex III1 and the III1/IV5 SC (Fig. 4A, arrows, and B and C). This observation suggests that complex I sequesters the limited amount of assembled complex III1 and the III1/IV5 SC available in sh-2 cells, similarly to what was reported for other assembly defects of the bc1 complex (18) or complex IV (17). The fact that we could observe free bc1 complex as well as the III1/IV5 SC in DDM-solubilized mitochondria from C11orf83-depleted cells (Fig. 3C) was probably due to the dissociation of the I/III1/IV SC under these extraction conditions. Taken together, our data indicate that the loss of C11orf83 induces a deficiency in bc1 complex assembly.

To further analyze this bc1 complex assembly deficit in C11orf83-depleted cells, we immunoblotted two other bc1 complex subunits: UQCRB and CYC1 (Fig. 4A). These subunits were chosen according to the literature which describes bc1 complex assembly in yeast. In the current model, the Qcr7p/Qcr8p/Cytp subcomplex, called the bc1 core complex, interacts sequentially with two other preformed subcomplexes, Cyt1p/Qcr6p/Qcr9p and Cor1p/Qcr2p, to form intermediate complexes. The late stages of bc1 complex assembly correspond to the incorporation of Rip1p and Qcr10p and the dimerization of the full complex (5, 6). Therefore, by selecting UQRC1 (human ortholog of Cor1p), UQCRB (human ortholog of Qcr7p), and CYC1 (human ortholog of Cyt1p), we can study the three potential subcomplexes involved in the early stages of bc1 complex assembly. Using antibodies against UQRCB and CYC1, we observed a clear decrease in the bc1 complex and the III1/IV SC in C11orf83-depleted cells, which is in agreement with the results obtained with the anti-UQRC1 antibody (Fig. 4A). At lower molecular weights, we noticed an additional accumulation of complex intermediates containing CYC1 but not UQCRB or UQCRB, presumably equivalent to the Cyt1p/Qcr6p/Qcr9p subcomplexes (Fig. 4A, arrow, and D). Importantly, overexpression of a C11orf83-V5 form (translated from
an mRNA which is not targeted by the shRNA) in sh-2 cells clearly decreased the accumulation of CYC1-containing intermediates (Fig. 4E and F). This result confirms that C11orf83 is an assembly factor involved in the early stages of bc1 complex assembly.

We next studied the effect of C11orf83 deletion on the steady-state levels of several bc1 complex subunits by performing immunoblotting on whole-cell lysates under denaturing conditions. We observed a pronounced reduction in the UQCRB (human ortholog of Qcr7p) (Fig. 5A and B) level and a slight reduction in the UQCRQ (human ortholog of Qcr8p) (Fig. 5A and C) level in C11orf83-depleted cells, suggesting a reduction in the amount of the bc1 core complex. The protein levels of all other analyzed subunits remained unchanged.

**FIG 3** C11orf83 depletion induces defects in the respiratory SC. (A) Western blot analysis of the steady-state levels of representative subunits of RC complexes (NDUFB2 for complex I [CI], SDHB for complex II [CII], UQCRCL for the bc1 complex [CIII], COX4 for complex IV [CIV], and ATP5B for complex V [CV]) in HeLa WT, sh-1, sh-2, and sh CTL cells. β-Catenin was used as a loading control. (B to E) Analysis of the assembly of RC complexes and SC. (B and C) DDM-solubilized isolated mitochondria from HeLa WT, sh-1, sh-2, and sh CTL cells were analyzed by BN PAGE and immunoblotting using antibodies against RC complexes. Differences between cell lines are shown by arrows. (D) Quantification of the I/III2/IV SC using antibody against NDUFB6 (mean ± SD [n = 3]). (E) Quantification of the III2/IV SC using antibody against UQCRCL (mean ± SD [n = 3]). *, P < 0.05; **, P < 0.01; ns, not significant.
C11orf83 Is Involved in bc₁ Complex Assembly

April 2015 Volume 35 Number 7
mcb.asm.org

FIG 4 C11orf83 is involved in bc₁ complex assembly. (A to D) Digitonin-solubilized isolated mitochondria from sh CTL and sh-2 cells were analyzed by 2D BN/SDS-PAGE. (A) The bc₁ complex was detected with antibodies against UQCRC1, UQCRB, and CYC1. Antibodies against NDUFV2, SDHB, and COX4 were used to detect complexes I, II, and IV, respectively. SDHB was used as a loading control since complex II assembly was shown to be unaffected by C11orf83 depletion and C11orf83 as a control for downregulation (Fig. 3B). Quantification of complex III₂ (B) and the III₂/IV SC (C) was performed using antibody against UQCRC1. Quantification of CYC1-containing complex intermediates at low molecular weights was performed using antibody against CYC1 (D). Values are means ± SDs (n = 3). (E) Digitonin-solubilized isolated mitochondria from sh CTL cells, sh-2 cells, and sh-2 cells transfected with C11orf83-V5 were analyzed by 2D BN/SDS-PAGE. The bc₁ complex was detected with antibodies against UQCRC1 and CYC1. The presence of endogenous C11orf83 or its V5-tagged form was detected by using an antibody against C11orf83. (F) Quantification of the CYC1-containing complex intermediates at low molecular weights using antibody against CYC1 (mean ± SD [n = 3]). *, P < 0.05.
specific phospholipid, are essential for the stabilization and function of the bc$_1$ complex (49) as well as for the stabilization of the III$_{b}$ IV$_{C}$ SC in yeast (50) and the III$_{b}$/IV and I/III$_{b}$/IV SC in mammalian cells (51, 52). As we have observed a bc$_1$ complex deficiency with an impaired formation of the III$_{b}$/IV SC in C11orf83-deficient cells, we hypothesized that C11orf83 could either be involved in phospholipid metabolism or interact with mitochondrial phospholipids. Therefore, we studied the lipid composition of mitochondrial-enriched fractions from either sh-2 or control cells by TLC. Mitochondrial fractions from both cell lines displayed similar levels of CL, phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC). They had equivalent CL-to-PE ratios, both when measuring TLC spot intensity (sh CTL, 0.71 ± 0.11; sh-2, 0.67 ± 0.13) and when measuring phosphate content (sh CTL, 0.23 ± 0.03; sh-2, 0.15 ± 0.05) (Fig. 6A). Quantification of total CL in both cell lines by MS confirmed the result (sh CTL, 1.4 ± 0.2 nmol/mg of protein; sh-2, 1.8 ± 0.4 nmol/mg of protein). CL has four acyl chains which can vary in length and unsaturation. Further analysis of mass spectra to evaluate CL acyl composition revealed that some CL species of the C16:0, C18:0, C18:1, and C18:2, C18:3, and C18:2, C18:3, C20:4, C20:5, C22:6, and C20:5, C22:6, and C20:5, C22:6, and C18:2, C18:3,) clusters were significantly increased in sh-2 cells (C16:0, C18:0, C18:1, C20:4, C20:5, C22:6, and C20:5, C22:6, and C18:2, C18:3,) clusters (Fig. 6B and C). However, there was no shift in unsaturation, only an increase in the abundance of the C16-enriched CL clusters. Our results indicate that the depletion of C11orf83 induces a shift in the CL composition of mitochondria to shorter acyl chains.

To analyze the potential binding of C11orf83 to mitochondrial phospholipids, we monitored the direct interaction of recombinant GST-C11orf83 protein on phospholipid blots (53). While recombinant GST did not produce any signal in this assay, GST-C11orf83 specifically bound to CL, PA, and sulfatide binding (Fig. 7A). C11orf83 binding to sulfatide, a sphingolipid, is most probably not physiologically relevant, as this lipid is not known to be present in mitochondria but is found in the plasma membrane in most eukaryotic cells (54). In contrast, C11orf83 may physiologically interact with CL and/or with PA, which is synthesized in the mitochondrial OM and transferred in the IM to be converted to CL in euukaryotic cells (55). To determine the CL-binding domain of C11orf83, we generated recombinant GST proteins corresponding to several subportions of C11orf83 and observed their potential interaction with phospholipids. GST-Δ23C11orf83, C11orf83 protein deleted of its N-terminal transmembrane domain, showed binding to CL, PA, and sulfatide similar to that shown by full-length C11orf83 (Fig. 7B and C), indicating that interaction with CL is not mediated by the C11orf83 membrane-anchoring domain. As most of CL-binding proteins interact with CL by their helices (56–59), we analyzed the phospholipid binding abilities of predicted C11orf83 helices, either separately (GST-helix 2 and GST-helix 3) or together (GST-helix 2-helix 3) (Fig. 7B and C). Fusion proteins GST-helix 2 and GST-helix 3 did not produce any signal in this assay, in contrast to the protein GST-helix 2-helix 3, which displayed a binding to CL, PA, and sulfatide similar to that of GST-C11orf83 (full length) (Fig. 7C). Taken together, our experiments showed that the combination of helix 2 and helix 3 (aa 23 to 80) is necessary and sufficient for CL binding (Fig. 7B) and could play a role in the CL binding of the bc$_1$ complex and/or in III$_{b}$/IV SC stabilization.

Recently, Cogliati and colleagues showed that crista morphology is linked to the assembly and stability of respiratory SC subunits were not modified compared to controls (Fig. 3A and 5A). Therefore, C11orf83 seems to be specifically involved in the stabilization of the bc$_1$ complex core at the early stage of bc$_1$ complex assembly. The accumulation of the CYC1-containing complex intermediates observed in the absence of C11orf83 (Fig. 4A) could be due to limiting amounts of the bc$_1$ complex core complex.

As we showed that C11orf83 is involved in bc$_1$ complex assembly, we wanted to check whether it interacts with this complex. A first indication came from the 2D BN/SDS-PAGE, in which C11orf83 was detected in bands migrating at the same molecular weights as complexes IV and III$_{b}$ and the III$_{b}$/IV SC (Fig. 4A). These observations indicate that C11orf83 could interact with bc$_1$ complex and with complex IV. To test this hypothesis, we conducted coimmunoprecipitation experiments. The bc$_1$ complex and complex IV were successfully immunoprecipitated from HeLa wild-type (WT) mitochondrial-enriched fractions solubilized using DDM, as shown by Western blot analysis using antibodies against UQCRFS1 and COX4, respectively (Fig. 5D). C11orf83 was specifically copurified with the bc$_1$ complex, which indicates that C11orf83 interacts with this complex. Under these conditions, we did not detect any interaction between C11orf83 and complex IV (Fig. 5D).

C11orf83 is a CL-binding protein involved in crista maintenance. Phospholipids, in particular CL, the mitochondrial-spe-
FIG 6 C11orf83 loss induces changes in fatty acid CL composition (A) TLC analysis of the lipid compositions of mitochondrion-enriched fractions from sh-2 or sh CTL cells. (B) Representative CL mass spectra from sh CTL and sh-2 cells. The different CL clusters are indicated using CXX (where the number "XX" designates the amount of carbon atoms in the fatty acid side chains). m/z 619.4 and m/z 665.4 are the CL and phosphatidylglycerol (PG) internal standards, respectively. (C) Quantification of CL species in sh-2 and sh CTL cells (mean ± SD [n = 4]). CL species are represented as CL(XX:Y), where XX designates the total amount of carbon atoms and Y corresponds to the total number of double bonds in the fatty acid side chains. There is a subtle but significant increase in the abundance of the C64, C66, and C68 clusters. *, P < 0.05; **, P < 0.01.
Since we observed defects in bc1 complex-containing SC in the absence of C11orf83, we suspected modifications in the IM morphology. Using electron microscopy, we confirmed a disorganization of the IM ultrastructure and cristae architecture in C11orf83-depleted cells compared to control cells (Fig. 8A). This observation is strikingly similar to what was reported with cells depleted for OPA1, a GTPase with the same localization and topology as C11orf83 (61–63). In addition, C11orf83-depleted cells present several features which are similar to those observed in OPA1-downregulating cells, including impaired respiration, decreased ETC enzymatic activities, and high apoptosis sensitivity (62, 63) (Fig. 2C and E; Table 1). Because OPA1 was shown to be involved in high-molecular-weight complexes (60), we analyzed whether C11orf83 depletion induced changes in mitochondrial OPA1-containing complexes. Figure 8B shows the migration pattern of OPA1 in C11orf83 depleted cells compared to sh control cells in 2D BN/SDS-PAGE. Whereas OPA1 was found in complexes of the expected size of around 480 and 720 kDa in control cells (60), OPA1 was aberrantly localized in complexes of various molecular masses in C11orf83-depleted cells. As OPA1 is located in the crista junction and forms a molecular bridge between the adjacent membranes of the cristae (64), the formation of aberrant complexes containing OPA1 may be due to the disorganization of the IM ultrastructure observed in C11orf83-deficient cells (Fig. 8B).

C11orf83 is cleaved by OMA1 upon mitochondrial stress, like OPA1. OPA1 is known to undergo proteolytic processing by several proteases, either constitutively or upon mitochondrial depolarization (65–68). For example, under stress conditions induced by CCCP, OPA1 is cleaved and inactivated by the zinc metalloprotease OMA1 (67, 68), which consequently promotes mitochondrial fragmentation. It was reported that C11orf83 was degraded upon exposure to CCCP in parkin-overexpressing cells (69). Therefore, we compared the proteolytic processing of C11orf83 and OPA1 in HeLa cells that do not overexpress parkin under different stress conditions that induce mitochondrial membrane potential (ΔΨ) loss, ATP loss, reactive oxygen species production, or transmembrane pH gradient (ΔpH) loss. We showed, by Western blot analysis, that both C11orf83-V5 and OPA1 are specifically degraded upon stresses that induce ΔΨ loss (CCCP, valinomycin, and FCCP) (Fig. 9A). We performed a protease inhibitor screening to identify the enzyme(s) responsible for C11orf83 degradation. C11orf83-V5 degradation was impaired by O-PHE, an inhibitor of metalloproteases, and to a lesser extent by DCI, an inhibitor of serine proteases (Fig. 9B). Since OMA1 is known to cleave OPA1 upon CCCP-induced stress, we downregulated OMA1 expression using siRNA and analyzed the proteolysis of C11orf83-V5 in the presence or absence of CCCP. In the absence of CCCP, the OMA1 siRNA did not modify the C11orf83-V5 protein level. However, upon CCCP stress, we observed that OMA1 downregulation was correlated with a reduction in C11orf83-V5 degradation (Fig. 9C). The residual C11orf83-V5 proteolysis might be due to residual OMA1 protein (around 20%) unaffected by the siRNA treatment or to DCI-sensitive serine proteases as hinted by the protease inhibitor screening assay. The same experiment performed on endogenous C11orf83 instead of overexpressed C11orf83-V5 confirmed that C11orf83 is a target of OMA1 upon mitochondrial depolarization (see Fig. SA3 in the supplemental mate-
In contrast to data reported by Head and colleagues, we were not able to recover the long OPA1 isoforms with siRNA against OMA1 (67). This observation can be explained by the incomplete OMA1 downregulation and/or by the absence of L-OPA1 overexpression in our experiment. Altogether, these data confirm that C11orf83, like OPA1, plays a key role in cristal structure maintenance by allowing proper assembly of the bc1 complex and SC stabilization and that it may be regulated by OMA1 activity.

**DISCUSSION**

In this study, we have identified C11orf83, a previously uncharacterized human protein, as an integral mitochondrial IM protein facing the IMS. We have established that the N-terminal part (aa 1 to 23) of this protein is responsible for its targeting and anchoring and that the basic residues at positions 5 and 6 are especially important. We have demonstrated that C11orf83 depletion in HeLa cells induced a decrease in ATP level due to impaired respiration. Although the enzymatic activities of the five RC complexes were found to be affected by this depletion, C11orf83 was shown to be specifically required for the early stages of bc1 complex assembly, probably by stabilizing the bc1 core complex. We observed that C11orf83 loss also induced a clear decrease in the abundance of the III2/IV and I/III2/IV SC and a disorganization of the mitochondrial ultrastructure. These two observations are probably linked, since crista morphology maintenance and SC formation have been shown to be connected (60). These mitochondrial ultrastructure defects may also explain the reduced enzymatic activities of all the ETC complexes, as reported for OPA1-depleted cells (63). A recent study highlighted that OPA1 oligomerization was required both to modulate crista ultrastructure and to regulate complex V assembly by stabilizing the expression of the F0 subunit MT-ATP6 (70). In C11orf83-deficient cells, OPA1-containing complexes were disturbed and complex V activity was decreased. Therefore, we can speculate that the lower complex V activity observed in the absence of C11orf83 may be due to a deficiency of the F0 subunit of complex V. In addition, we demonstrated that C11orf83 depletion led to a higher sensitivity to actinomycin D-induced apoptosis. Since the remodelling of the cristae seems to be a prerequisite to apoptosis (71), we suspect that this may also be a consequence of the IM destabilization. Finally, since crista alteration and reduced RC activity were reported to occur in aged cells (42–46, 72), we suspect that mitochondrial dysfunctions observed in C11orf83-depleted cells could explain their growth deficit after several passages.

We have shown that the OMA1 metalloprotease mediates the cleavage of C11orf83 in response to mitochondrial depolarization. Given our results for C11orf83-depleted cells, we can hypothesize...
that the specific proteolysis of C11orf83 by OMA1 in cells with depolarized mitochondria may destabilize the respiratory SC, inducing cristae disorganization and increasing their sensitivity to apoptosis. To our knowledge, there is no other example of an assembly factor or a respiratory complex subunit which would be a target of OMA1 proteolysis upon stress. In contrast, it is well known that OPA1 is cleaved by OMA1 upon mitochondrial depolarization, like C11orf83, and that this induces mitochondrial fission and cristae disorganization, resulting in enhanced apoptosis sensitivity (67, 68, 73).

During our study, we found a specific interaction of C11orf83 with the \( bc_1 \) complex. However, since we observed that C11orf83 comigrated not only with the \( bc_1 \) complex and III/IV SC in native gels but also with complex IV, we cannot exclude a transient interaction of C11orf83 with complex IV. A similar case has been recently described for \( S. cerevisiae \): Rcf1p, identified both as an assembly factor for complex IV and as a stabilizing factor of the III/IV2 SC (21), stably interacts with complex IV and also weakly binds to the \( bc_1 \) complex (74). Furthermore, since we did not detect C11orf83 in the I/III/IV SC complex, we propose that C11orf83 could be involved in the stabilization of the III/IV SC and be removed when this smaller SC associates with complex I. The formation of the I/III/IV SC could stabilize the interaction between complexes III2 and IV, rendering C11orf83 stabilizing function redundant and leading to the elimination of this protein from the SC. In the literature, the complex I assembly chaperone mimmitin (NDUFAF2) is also described as being transiently used for a specific step of assembly and then released to allow assembly completion (75).

Finally, we showed that C11orf83 was able to bind to CL and to its precursor PA (76). CL is an important IM phospholipid involved in the stabilization of individual ETC complexes and SC (49, 51, 52, 77). Several phospholipid-binding sites were described for the \( bc_1 \) complex and complex IV, among which some are specific to CL (56, 57, 78). For the \( bc_1 \) complex, most of these CL sites were identified on the matrix side of the IM bilayer or in the transmembrane domains of proteins like Cyt1p or Cytbp (59, 78). However, interactions of CL with ETC complexes on the IMS leaflet of the IM were previously reported for cytochrome c (79) and for complex IV (57) and might be involved in the formation and stabilization of the III/IV SC (57). Similarly, we propose that the CL-binding properties of the IMs-protruding C11orf83

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**FIG 9** C11orf83, like OPA1, is cleaved by OMA1 under \( \Delta \Psi \) loss. (A) Analysis of the stress-induced proteolysis of C11orf83 compared to OPA1. HeLa cells expressing C11orf83-V5 were treated with mitochondrion-damaging agents for 3 h, and cell lysates were analyzed by immunoblotting after SDS-PAGE. \( \beta \)-Catenin and SDHB were used as loading controls for cytosolic and mitochondrial proteins. Stress-induced cleavage of OPA1 and C11orf83 was observed by antibodies against OPA1 and the V5 tag. ROS, reactive oxygen species. (B) Analysis of the sensitivity of CCCP-induced cleavage of C11orf83 to various protease inhibitors. HeLa cells expressing C11orf83-V5 were incubated with CCCP with or without protease inhibitors. Cells lysates were fractionated by SDS-PAGE and analyzed by immunoblotting. \( \beta \)-Catenin and SDHB were used as loading controls. The prevention of C11orf83-V5 proteolysis was monitored thanks to an antibody against the V5 tag. (C) OMA1 involvement in the stress-induced cleavage of C11orf83. HeLa cells were transfected with C11orf83-V5 and treated with control siRNA or siRNA against OMA1. Forty-eight hours after siRNA transfection, cells were incubated with or without CCCP and cell lysates were analyzed by immunoblotting, after SDS-PAGE, using antibodies against OMA1, OPA1, and the V5 tag. GAPDH was used as a loading control. The two bands detected by the antibody against OPA1 in the absence of CCCP correspond to short and long OPA1 isoforms. The degradation of endogenous C11orf83 under CCCP treatment (lane 3) was prevented by the depletion of OMA1 (lane 4).
would be involved in the stabilization of III/IV SC, which might explain the reduction of the abundance of III/IV SC observed after C11orf83 depletion. We found that the CL binding of C11orf83 did not require its N-terminal part, responsible for IM targeting and anchoring, but required both α-helices of the IMS part, indicating that they provide a unique tertiary structure suitable for the interaction with CL. Similar observations were reported for the truncated proapoptotic protein tbid, whose CL-binding domain encompasses three helices, H4, H5, and H6 (80). Studies on the composition of the CL binding sites of the RC complexes highlighted that the side chains of positively charged amino acids (lysine and arginine) were frequently involved in electrostatic bonds with CL due to CL’s negative charge (57, 78, 81). Phenylalanine and leucine residues were also found to be involved in CL binding sites of complexes I and III (57). In addition, amino acids which contribute to CL binding are often well conserved over species (78). According to these data, we introduced several mutations in GST-C11orf83 (L24A, K35A, K40A, R55A, and R87A) and assessed the CL binding of these mutated forms. Unfortunately, none of these point mutations impaired the CL binding ability of C11orf83 (data not shown). Additional structural investigations by X-ray crystallography coupled to extensive site-directed mutagenesis studies would be needed to characterize the interaction of C11orf83 with CL at the molecular level.

We noticed that C11orf83-deficient cells presented some features similar to those observed following depletion of proteins involved in CL synthesis. HeLa cells downregulating the CL synthase showed an increased susceptibility to cell death due to an accumulation of free cytochrome c in the IMS of mitochondria with disorganized cristae (82). In these CL synthase-downregulating cells, the level of CL is still detectable and sufficient to sustain a normal OXPHOS. In contrast, lymphoblasts from patients with Barth syndrome (BTHS) (83), a genetic disease caused by mutations in the TAZ gene encoding tafazzin, an enzyme involved in CL maturation, showed severe mitochondrial respiratory deficiencies due to a destabilization of respiratory SC, reduced enzymatic activities of complexes I, III, and IV, and defects in mitochondrial ultrastructure (52, 84). In addition, tissues of BTHS patients and TAZ-deficient cells revealed accumulation of monolysoc-LCL (a remodeling intermediate) and an abnormal composition of CL showing a shift to less saturated acyl chains (85, 86). These changes in CL composition are thought to perturb the mitochondrial membrane and cause the disorganized cristae. Although we showed that the total amount of CL was not altered in C11orf83-deficient cells, we did observe a subtle but significant increase in CL species enriched in C16 fatty acids. The CL acyl chain composition in general is known to vary between organisms and between tissues and cell types within the same organism (87, 88). These differences are thought to have a structural role in membranes and/or allow adaptation against environmental stress (88). Therefore, the changes observed in CL fatty acid composition in the absence of C11orf83 could represent an adaptation of the cell to cope with disorganized cristae and an impaired OXPHOS.

In summary, we identified and characterized C11orf83 as a new factor required for the assembly of the bc1 complex and for the stabilization of SC, which binds CL and is specifically cleaved by OMA1 upon stress. No obvious ortholog of C11orf83 was found by BLAST analysis in lower eukaryotes. However, a literature review indicated that S. cerevisiae Cbp4p, compared to C11orf83, has the same mitochondrial localization and topology, with an hydrophobic α-helix of around 20 aa (23 for C11orf83 and 22 for Cbp4p) at its N terminus involved in mitochondrial membrane targeting and anchoring (89) and a C-terminal part exposed in the IMS. In addition, C11orf83-deficient human cells and cbp4Δ mutant yeast strains display identical accumulations of YC1 CYt1p-containing subcomplexes and similarly reduced steady-state levels of UQCRB/UQCP7 and UQCRQ/UQR8p, indicating that Cbp4p and C11orf83 have similar implications in the early stages of bc1 complex assembly (90). Therefore, C11orf83 may be the functional homolog of Cbp4p in humans. During the writing of the manuscript, Wanschers and colleagues proposed that C11orf83, which they renamed UQCC3, would be the human ortholog of Cbp4p. They identified a missense mutation in C11orf83 (Val20Glu) that resulted in the destabilization of the protein in a patient and caused a bc1 complex deficiency (91). In addition to these findings, we have demonstrated that C11orf83 interacts with the bc1 complex. Although Kronekova and Rödel observed that Cbp4p-containing complexes comigrated with complex III, and weakly with the III/IV SC (90), no interaction between Cbp4p and either the bc1 complex or complex IV had yet been reported. We also demonstrated that C11orf83 is involved in the SC stabilization and binds to CL, two important features which had never been studied in yeast. Importantly, Wanschers and colleagues reported that cbp4Δ mutant yeast strains could not be complemented by C11orf83 (91). This result could be due to a low level of sequence similarity between Cbp4p and C11orf83. For example, the RK motif, which we found to be essential for C11orf83 mitochondrial localization (Fig. 1A, E, and H), is present in higher eukaryotes but not in yeast. These variations of sequence may explain the not fully overlapping properties observed between Cbp4p and C11orf83, reflecting the mitochondrial ETC evolutional differences between S. cerevisiae and Metazoa.

ACKNOWLEDGMENTS

We thank Denis Martinvalet, Oliver Hartley, Maureen Deegan, Paula Duck, Rachel Porcelli, Marianne Paolini-Bertrand, and Irène Rossitto-Borlat for comments and suggestions, review of the manuscript, and technical assistance, and we thank Mathias Uhlen (Human Protein Atlas, Sweden) for access to transmission electron microscopy equipment and M. A. T. Vervaart, Laboratory Genetic Diseases, Amsterdam, The Netherlands, for performing the CL MS analysis. This work was supported by the Faculty of Medicine of the University of Geneva, the Swiss National Science Foundation (grant no. 310030 152618 and CRSII13–141798 to M.F.), and the Ernst and Lucie Schmidheiny Foundation (grant to M.D.).

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