**MR-1S Interacts with PET100 and PET117 in Module-Based Assembly of Human Cytochrome c Oxidase**

**Graphical Abstract**

**Highlights**
- Human cytochrome c oxidase (COX) assembles in a modular rather than sequential fashion
- HIGD1A is associated to COX and is assembled in the initial phase
- MR-1S, the smallest product of PNKD, is a vertebrate-specific COX assembly factor
- MR-1S interacts with the highly conserved COX chaperones PET100 and PET117

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**In Brief**
By quantitatively analyzing the COX intermediates accumulated in a MT-CO3 mutant, Vidoni et al. modify the existing sequential COX assembly model to a module-based pathway and describe MR-1S as a vertebrate-specific factor involved in COX maturation, cooperating with the highly conserved PET100 and PET117 chaperones.

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MR-1S Interacts with PET100 and PET117 in Module-Based Assembly of Human Cytochrome c Oxidase

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SUMMARY

The biogenesis of human cytochrome c oxidase (COX) is an intricate process in which three mitochondrial DNA (mtDNA)-encoded core subunits are assembled in a coordinated way with at least 11 nucleus-encoded subunits. Many chaperones shared between yeast and humans are involved in COX assembly. Here, we have used a MT-CO3 mutant cybrid cell line to define the composition of assembly intermediates and identify new human COX assembly factors. Quantitative mass spectrometry analysis led us to modify the assembly model from a sequential pathway to a module-based process. Each module contains one of the three core subunits, together with different ancillary components, including HIGD1A. By the same analysis, we identified the short isoform of the myofibrillogenesis regulator 1 (MR-1S) as a new COX assembly factor, which works with the highly conserved PET100 and PET117 chaperones to assist COX biogenesis in higher eukaryotes.

INTRODUCTION

Cytochrome c oxidase (COX) or complex IV (cIV) is the multiheteromeric terminal oxidase of the mitochondrial respiratory chain. Of the 14 structural subunits composing mammalian COX, three (MT-CO1, CO2, and CO3) are encoded in the mitochondrial DNA (mtDNA) and translated inside the organelle. The remaining 11 subunits and all of the other proteins assisting the assembly process are imported into the organelle from the cytoplasm. More than 30 different ancillary proteins are required for the correct assembly of the 11 structural subunits of yeast COX (Soto et al., 2012). Several orthologs have been found in humans, mainly because their mutations determine COX defective mitochondrial disease (Antoniccka et al., 2003a, 2003b; Bugiani et al., 2005; Jaksch et al., 2000; Leary et al., 2004; Lim et al., 2014; Oláhová et al., 2015; Szkarczyk et al., 2013; Volny et al., 2000a, 2000b). The current assembly model for human COX (Nijtmans et al., 1998; Stiburek et al., 2005, 2006) consists of four steps: early (step 1 [S1]), intermediate (S2), late (S3), and last (S4). MT-CO1 is deemed as the first COX component to be inserted in the inner mitochondrial membrane and constitute the “seed” onto which the other subunits are assembled (S1). The incorporation of two early subunits, COX4 and COX5A, with MT-CO1 forms the S2 intermediate. This is followed by the incorporation of subunits MT-CO2 and then MT-CO3, which in turn triggers the “late” incorporation of most of the remaining accessory subunits (S3), with the exception of COX6A, COX6B, and COX7A or COX7B, which are incorporated last (S4) (Fernandez-Vizarra et al., 2009). The mechanisms and players involved in this process are relatively well established in yeast (Mick et al., 2011), whereas the process is less clear and possibly different in mammals (Dennerlein and Rehling, 2015; Soto et al., 2012). For instance, specific factors present in mammals but not in yeast are necessary for the incorporation of MT-CO1 (Clemente et al., 2013; Dennerlein et al., 2015; Mick et al., 2012) and for stabilization and metalation of MT-CO2 (Bourens et al., 2014; Leary et al., 2009; Pacheu-Grau et al., 2015; Stroud et al., 2015).

In order to further define the key steps in COX assembly and identify new players in human COX biogenesis, we took advantage of a nearly homoplasmic frameshift mutation in MT-CO3 (Tiranti et al., 2000), characterized by the accumulation of assembly intermediates.

RESULTS

Analysis of Assembly Intermediates Recapitulates COX Biogenesis

Fragment length polymorphism (RFLP) analysis on a cybrid cell line harboring the m.9536_9537insC (p.Gln111Profs*113) mutation (Tiranti et al., 2000) in MT-CO3 showed that the mutation load was >95% (Figure S1). By western blot (WB) analysis of dodecyl-maltoside (DDM)-treated mitochondrial fractions,
run through first-dimension (1D) blue native gel electrophoresis (BNGE), we detected numerous MT-CO1-containing subassembly species (Figure 1A), which were likely to contain chaperones involved in COX assembly (Dennerlein and Rehling, 2015). In order to characterize the composition of the subassembly COX species, we carried out a comparison of COX immunopurified from mitoplasts of wild-type (WT) and cybrid mutant cell lines by quantitative mass spectrometry (MS) analysis using stable isotope-labeled amino acids in cell culture (SILAC). These experiments were performed in duplicate with reciprocal isotopic labeling between mutant and WT cell lines and showed that the COX subunits were clustered in four distinct groups according to their relative abundance (Figure 1B; Table S1), in nearly complete concordance with the current COX assembly model (Fernández-Vizarra et al., 2009; Fornuskova et al., 2010; Nijtmans et al., 1998; Stiburek et al., 2006). Interestingly, the first group included COX4I1, COX5A, and HIGD1A, a protein previously proposed to bind MT-CO1 and regulate COX activity in hypoxia (Hayashi et al., 2015). The next group was composed of proteins with ~3.5-fold reduction in the mutant cell line and included MT-CO2, COX5B, COX6C, COX7C, COX8A, and, unexpectedly, MT-CO1. The third group, with more than 5-fold difference between mutant and WT complexes, included MT-CO3, COX6A1, COX6B1, and COX7A2. The last group contained COX7A2L (also termed super-complex assembly factor 1 [SCAF1]) (Lapuente-Brun et al., 2013), NDUFA4 (Balsa et al., 2012; Pitceathly et al., 2013), and virtually all of the subunit components of complex III (Figure 1B; Table S1). These results reflect the stalling of COX assembly due to the virtual absence of MT-CO3 in the mutant cell line, which prevents the formation of cIII$_2$ + cIV supercomplexes and causes the accumulation of COX assembly intermediates.
These assembly intermediates were also analyzed by “complexome profiling” (Heide et al., 2012). Proteins from mutant and WT cybrid cells were labeled with SILAC, combined and resolved by 1D-BNGE, and excised in 64 slices (Figures 2A and 2B). Figure 2A shows the heatmap of WT mitochondria in comparison with the mutated mitochondria. In WT mitochondria, COX subunits are focalized in slice 39 (at a molecular size of 215 kDa), whereas they are much more scattered in the MT-CO3 mutated cells. The profiles for the individual subunits (Figure 2B) indicate that subunits COX4I1 and COX5A (not shown), as well as HIGD1A, are distributed in the bottom part of the gel (from 30 kDa upward), thus representing early subcomplexes. In mutant mitochondria, these subunits are predominantly found in assembly intermediates rather than in (residual) fully assembled COX. The “intermediate” group of subunits was detected from higher positions along the gel (from 120 kDa upward), and again, in the mutant cell line, they were predominantly found in subassemblies rather than in residual fully assembled COX. Interestingly, the intermediate group included MT-CO1, suggesting that in mammalian cells, the incorporation of this subunit occurs at a stage later than the initial one, which instead corresponds to the formation of the COX4I1, COX5A, and HIGD1A cluster. Finally, the last subunits to be incorporated were exclusively present in fully assembled COX, and their relative amount was virtually negligible in the mutant cell line.

**Identification of a Novel COX Assembly Factor**

In order to identify COX-specific assembly factors associated with assembly intermediates, we sought proteins associated with mutant COX assemblies in greater abundance than with WT cybrid lines. In a cluster of proteins with mutant/WT ratios of ~1.5–2, four were already known to participate in COX biogenesis, namely,
COX3 (CCDC56 or MITRAC12) (Clemente et al., 2013; Mick et al., 2012), PET100 (Lim et al., 2014; Oláhová et al., 2015; Szklarczyk et al., 2012), and the human orthologs of the yeast Pet117 (McEwen et al., 1993; Szklarczyk et al., 2012) and Cmc2 (Horn et al., 2010). In this cluster, we identified a fifth protein, myofibrillary-related protein 1 short isoform (MR-1S; also known as PNKD isoform 2; Uniprot: Q8N490-2), which we investigated further as a potential, new candidate assembly factor (Figures 1B and 1C).

The migration pattern of MR-1S-containing assembly intermediates was determined in reciprocal complexome analyses with the "empty" vector or with an shMR-1SRNA1 clone, whereas MT-CO1, MT-CO2, COX5A, and COX5B, but not COX6B, were present in the immunopurified material from MR-1SHA-expressing cells (Figure S3A). In mtDNA translation experiments, MR-1SHA was able to bind newly synthesized MT-CO2 and, in smaller proportions, MT-CO1 as well (Figure S3B).

To further explore the MR-1S-specific interactome, we examined the protein interactions of HA-tagged MR-1S by MS of SILAC-labeled α-HA immunoprecipitates from both WT and MT-CO3 cybrids. The specific interactors consistently detected by these analyses included (1) several COX structural subunits belonging to the early or intermediate assembly groups and (2) two COX assembly factors, i.e., PET100 (Church et al., 2005; Lim et al., 2014; Oláhová et al., 2015) and PET117 (McEwen et al., 1993; Soto et al., 2012; Szklarczyk et al., 2012) (Figures 4E and 4F).

MR-1S-PET100 Complexes Identified by Complexome Profiling
To gain further insight on the physiological interactions of MR-1S, we performed complexome profiling of mitochondria isolated from 143B cells and C-124 human skin fibroblasts (Figures 5A and 5B). In both cell types, HIGD1A was detected in all blue native (BN) gel slices where COX subunits were present (i.e., in the monomeric COX, cI, cII, and cIV supercomplex and in the larger COX-containing supercomplexes), qualifying HIGD1A as a constitutive subunit of the COX complex (Figure 5B). Both MR-1S and PET100 were predominantly detected in the region around the COX holocomplex (214 kDa). Again, both their migration profiles showed two clear peaks at ~160 kDa and 270 kDa, which were distinctly different from that of the COX monomer (Figure 5B), indicating that they were bound to COX assembly intermediates.

The migration profiles also revealed that the putative assembly factor PET117 was present in a peak overlapping the COX holocomplex but showing a “shoulder” coinciding with the higher-molecular-weight peak containing MR-1S and PET100 (Figure 5B). This suggests an interaction between PET117 and MR-1S plus PET100 during COX biogenesis.

MR-1S Interacts with PET100 and PET117
The absence of human PET100 is associated with severe, isolated COX deficiency and impaired COX assembly, leading to...
a fatal early-onset mitochondrial encephalopathy (Lim et al., 2014; Oláhová et al., 2015). The role of human PET117 in COX biogenesis is deemed to be similar to that shown in yeast, although no experimental confirmation has thus far been provided in mammalian systems (Szklarczyk et al., 2012). In fibroblasts carrying a truncating homozygous p.Gly48* mutation in PET100 (Oláhová et al., 2015), the MR-1S steady-state levels were markedly reduced (Figure 6A) and the typical MR-1S-containing supramolecular structures were absent (Figures 6B and SS), suggesting that the stability and interaction of MR-1S with COX assembly intermediates depend on the presence of PET100.

Next, we transduced WT cybrids, control, and PET100G48* immortalized fibroblasts transduced with FLAG-tagged versions of either PET100 or PET117. All cell lines showed very high expression of recombinant PET117FLAG whereas the levels of PET100FLAG were much lower (Figure 6C) but sufficient to rescue the COX assembly and activity (Figure S4) and (partially)
complement the molecular phenotype of PET100 G48* fibroblasts (Figure 6A).

In SILAC experiments using naive WT cybrids and cybrids transduced with PET117FLAG, MR-1S was co-immunoprecipitated by an α-FLAG monoclonal antibody, together with early (COX4I1 and COX5A) and intermediate (MT-CO1, MT-CO2, COX5B, and COX6C) COX structural subunits and COX11, another COX assembly factor (Figure 6D). More work is necessary to establish whether COX11 and MR-1S co-operate in COX assembly. Likewise, in PET100 G48* immortalized fibroblasts transduced with PET100FLAG, the α-FLAG antibody co-immunoprecipitated MR-1S, together with MT-CO1, MT-CO2, COX5A, and COX5B (Figure 6E). However, no co-immunoprecipitation of any of these proteins was obtained in the PET100 G48* fibroblast cell line transduced with PET117FLAG (Figure 6E). Taken together, these results clearly demonstrate that PET117 physically interacts with MR-1S and with some COX subunits and that this interaction requires the presence of PET100.

DISCUSSION

Revisiting the COX Assembly Pathway

Both immunocapture/SILAC and complexome/SILAC analyses converge in defining four groups of subunits (early, intermediate, late, and last) differentially clustering in subassembly intermediates during COX biogenesis.
The first group includes COX4I1 and COX5A subunits, which were proposed to be the first to join MT-CO1 and determine the transition from assembly step 1 (S1, corresponding to monomeric MT-CO1) to S2 (Figure 7A). Accordingly, these two subunits were found in several early, low-molecular-weight subcomplexes. In immunoprecipitation and SILAC experiments, HIGD1A, which is proposed to mainly interact with MT-CO1 (Hayashi et al., 2015), appeared with the same relative abundance in the MT-CO3 mutant versus WT cells and in low-molecular-weight subcomplexes, like the subunits COX4I1 and COX5A. However, in complexome experiments, HIGD1A also co-migrates with higher-order COX structures (from 300 to 500 kDa) in a non-stoichiometric proportion compared to the other COX subunits (Figure 2B). HIGD1A is a mammalian ortholog of Rcf1, a COX-interacting factor that helps stabilize the cIII2 + cIV (1-2) supercomplexes in yeast (Chen et al., 2012; Strogolova et al., 2012; Vukotic et al., 2012), and when bound to COX, it increases its activity (Hayashi et al., 2015; Rydstrom Lundin et al., 2016). Thus, HIGD1A may be a regulatory component of COX, which is incorporated in the early stages of the enzyme assembly.

Unexpectedly, both the immunoprecipitation/SILAC and complexome analyses showed MT-CO1 to be part of the intermediate assembly group of COX subunits rather than the early-assembly group including COX4I1 and COX5A. Previous studies have shown that the knockdown of COX4I1 expression results in a similar decrease in the COX5A steady-state levels and vice versa. In addition, the levels of fully assembled COX were the same as the residual levels of either subunit (Fornuskova et al., 2010). This is consistent with the idea that these subunits interact and play a seminal role in the first stages of COX assembly. Importantly, the total amount of MT-CO1 was unchanged, but there was an accumulation of MT-CO1-containing assembly intermediates. These findings support the idea that shortage of the COX4I1 + COX5A early subassembly impairs the incorporation of MT-CO1, leading to accumulation of MT-CO1 intermediates, concordant with the idea of a separate MT-CO1 module. This later incorporation of MT-CO1 was also previously suggested in S. cerevisiae (Church et al., 2005), and is in agreement with kinetic experiments of mitochondrial translation after doxycycline treatment (Moreno-Lastres et al., 2012).

Our complexome profiling confirmed that the intermediate assembly group includes, in addition to MT-CO1, the subunits MT-CO2, COX5B, COX6C, COX7C, and COX8A. In the current model, the incorporation of MT-CO3 occurs just before or concomitantly with the intermediate group of subunits (Figure 7A). However, our data indicate that the incorporation of the nucleus-encoded accessory subunits (COX5B, COX6C, COX7C, and COX8A) parallels that of MT-CO2 and MT-CO1 and occurs before, rather than after, the incorporation of MT-CO3 (Figures 1, 2, and 7B).

In fact, our data indicate that the late assembly group includes subunit MT-CO3, together with COX6A1, COX6B1, and COX7A2. The “last” group includes two proteins: NDUFA4,
which is peripherally associated with COX (Balsa et al., 2012; Kadenbach and Huttemann, 2015; Pitceathly et al., 2013), and COX7A2L, which is necessary for the formation of cIII 2 + cIV (Cogliati et al., 2016; Pérez-Pérez et al., 2016; Williams et al., 2016). The incorporation of COX6B1 and COX6A1 in the late assembly group is in agreement with data obtained from the analysis of COX6B1 mutant human fibroblasts (Massa et al., 2008) and knockdown expression of COX6A1 in human cells (Fornuskova et al., 2010). In addition, our results confirm that COX7B (the non-muscular isoform of COX7A), rather than COX7B (Nijtmans et al., 1998; Stiburek et al., 2006), belongs to the late assembly group (Fornuskova et al., 2010) (Figure 7A). We have evidence that COX7B could in fact be incorporated with the intermediate assembly group (data not shown).

The current human COX assembly model is based on the sequential incorporation of individual subunits into a progressively bigger supramolecular structure. However, a modular assembly model has recently been suggested for the yeast

Figure 6. PET117 Interaction with MR-1S and COX Subunits Is Mediated by PET100

(A) Steady-state levels of proteins visualized by SDS-PAGE, WB, and immunodetection in the PET100G48* patient and in control (WT) immortalized fibroblasts transduced with PET100FLAG. The proteins were detected with the indicated antibodies. The signal intensities expressed as percentage of the control (WT, EV) of MT-CO2, MT-CO1, and MR-1S normalized to tubulin are plotted in the top graph (mean ± SD; n = 2). The signal intensities expressed as percentage of the control (WT, EV) of MT-CO2 and MT-CO1 normalized to the MR-1S signal are plotted in the bottom graph (mean ± SD; n = 2). See also Figure S4.

(B) 1D-BNGE, WB, and immunodetection analysis showing the absence of fully assembled COX, detected with α-MT-CO2, and of the MR-1S supramolecular complexes in the PET100G48* primary fibroblasts. See also Figure S5.

(C) SDS-PAGE, WB, and immunodetection using α-FLAG. Actin and tubulin (TUB) are loading standards. Notice that a long exposure was necessary to visualize the PET100FLAG band.

(D) Scatterplots and heavy-to-light (H/L) ratio table obtained after mass spectrometry analysis of α-FLAG co-immunoprecipitation fractions of bi-directional SILAC labeling of cybrid cells expressing recombinant PET117FLAG, combined with material from empty vector cybrid cells. Only the top right quadrant of the plot and the statistically significant interactions are shown (significance B, Perseus analysis platform; Cox and Mann, 2008; Tyanova et al., 2016).

(E) SDS-PAGE, WB analysis, and immunodetection of the co-immunoprecipitation fractions using α-FLAG in PET100 G48* immortalized fibroblasts overexpressing PET100FLAG or PET117FLAG. TOT, total mitoplast lysate before co-immunoprecipitation. FT, flow-through fraction with the unbound proteins. WASH1 and WASH9, fractions obtained after the first and ninth washings of the α-FLAG-M2-agarose resin. ELUTE, eluted fractions of the material bound to the resin after treatment with the specific FLAG peptide.
Likewise, in human cultured cells, MT-CO1 was found to participate in an assembly module termed MITRAC (for mitochondrial translation/regulation of assembly intermediates of COX) (Dennerlein et al., 2015; Mick et al., 2012), together with COX structural subunits and the assembly factors COA3 (MITRAC12), COA1 (C7orf44 or MITRAC15), COX14 (C12orf62), CMC1, and SURF1. In yeast, a second module contains Cox3 together with Cox4 (ortholog of human COX5B), Cox7 (COX7A), and Cox13 (COX6A). A third module consists of Cox2 together with other yet-to-be-determined subunits (Su et al., 2014). These three modules then join together, release the chaperones, and give rise to mature COX. The heavy/light ratios obtained in our SILAC analyses support the existence of assembly modules rather than sequential incorporation, where MT-CO2 would form a module with at least COX6C, COX7C, COX8A, and COX5B, while MT-CO3 would go together with COX6A1, COX6B1, and COX7A2 (Figure 7 B).

**MR-1S Role in COX Assembly and Interaction with PET117 and PET100**

We found MR-1S to interact with COX-related proteins. Accordingly, MR-1S binds to a subassembly species composed of the structural subunits defining the “S3” intermediate in the COX assembly model, except for MT-CO3. Drastic knockdown of MR-1S expression had functional consequences on COX activity and respiratory function, confirming its involvement in COX biogenesis. A role in the intermediate assembly steps of COX is also supported by the fact that the advanced intermediates and fully assembled COX are less abundant in the MR-1S knockdown cells.

**Figure 7. New Complex IV Assembly Model versus the Reference Assembly Model**

(A) Reference assembly model based on Nijtmans et al. (1998); Stiburek et al. (2005), (2006).

(B) New modular assembly model. Each of the modules corresponds to one of the mtDNA-encoded subunits (MT-CO1, MT-CO2, and MT-CO3). The MT-CO1 module is labeled as the MITRAC complex (Mick et al., 2012). Proteins whose genes have been found mutated in mitochondrial disorders are displayed in red.
Mutations in exon 1 of the *PNKD* gene, encoding the mitochondrial targeting sequence of MR-1S (S for short) and MR-1L (L for long), have been associated with the autosomal-dominant neurological disease paroxysmal non-ketogenic dyskinesia (PNKD) (Ghezzi et al., 2009). In contrast with the ubiquitous distribution of MR-1S, MR-1L is only expressed in the brain. Interestingly, low OCR was found by Seahorse analysis in patient fibroblasts carrying the A9V mutation (Ghezzi et al., 2015), but the molecular pathogenic mechanisms of PNKD remain elusive, since the mutation does not affect the mature forms of both MR-1S and MR-1L. The function of both MR-1L, which localizes to mitochondria, and MR-1M, which localizes to the Golgi apparatus and endoplasmic reticulum (ER), remains unknown but is likely not related to the COX-specific chaperone role that we have demonstrated for MR-1S.

Our data show that MR-1S co-immunoprecipitates with PET100, a known COX assembly factor (Church et al., 2005; Lim et al., 2014; Oláhová et al., 2015), and vice versa. Yeast Pet100 is necessary for COX assembly and forms a subcomplex with Cox7 (COX7A), Cox7a (COX6C), and Cox8 (COX7C) (Church et al., 1996, 2005). Interestingly, two of the human ortholog subunits (COX6C and COX7C) were present in the same fractions as PET100 when MR-1SHA was immunoprecipitated.

Human skin fibroblasts deficient in PET100 show profoundly reduced COX levels (Lim et al., 2014; Oláhová et al., 2015). PET100G48* mutant cells, MT-CO2 is drastically reduced, whereas MT-CO1 is also low, but to a lesser extent (Oláhová et al., 2015; Figure 6A). In PET100G48* mutant fibroblasts (Lim et al., 2014), the turnover of MT-CO2 and MT-CO3 was much higher than in control cells. This is totally consistent with the idea that PET100 is important for the formation and/or stabilization of the intermediate lacking the MT-CO3 module, which would be generated when the COX4I1 + COX5A and the MT-CO3 + MT-CO2 modules get together, preceding the incorporation of the intermediate lacking the MT-CO3 and its partners. This is the same step in which we propose that MR-1S binds the COX assembly intermediates.

In addition to PET100, MR-1S interacts with PET117, whose involvement in COX assembly in human cells was never described before. By using PET100-defective cells (PET100G48*), we were able to determine that the interactions among MR-1S, PET117, and COX structural subunits require PET100. In contrast with PET100, PET117, and many other COX assembly factors, MR-1S is present only in vertebrates, suggesting for this protein a peculiar function that is probably specific to the higher complexity and plasticity of COX in these organisms compared to that in yeast or non-vertebrate pluricellular eukaryotes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

Human 143B osteosarcoma cells and primary and immortalized skin fibroblasts were grown at 37°C and 5% CO2 in high-glucose plus Glutamax and sodium pyruvate DMEM (Life Technologies) supplemented with 10% fetal bovine serum (FBS), penicillin and streptomycin, and 50 μg/mL uridine. For puromycin and hygromycin, concentrations were 1 μg/mL and 100 μg/mL, respectively.

**Cell Line Generation**

Primary skin fibroblasts were immortalized by lentiviral transduction of pLOX-Ttag-iresTK (Addgene #12246, Tronolab).

**C-terminal HA or FLAG tags were added by PCR amplification. PCR fragments were cloned into pWPXLd-ires-Puro6 and pWPXLd-ires-Hygro6 lentiviral expression vectors, modified versions of pWPXLd (Addgene #12258, Tronolab). Lentiviral particles were generated in HEK293T packaging cells, and target cells were transduced as described previously (Perales-Clemente et al., 2008). 24 hr after transduction, cells were selected for puromycin or hygromycin resistance.

RNAi was performed by lentiviral transduction of shRNA sequences targeting specifically the MR-1S isoform (target 1, 5′-GGGATTCGCTACTTATTCTCCA TCC-3′; target 2, 5′-GGCAGCGCAAGGCGAAATAAG-3′; and target 3, 5′-GGA TGATAGCTGTTAAAGT-3′), cloned into the pLKO.1-TRC vector (Addgene #10878) according to the manufacturer’s instructions (https://www.addgene.org/tools/protocols/plko/).

**Native and Denaturing Protein Electrophoresis and WB**

Samples for BNGE were prepared as described previously (Nijtmans et al., 2002; Wittig et al., 2006). Native samples were run through pre-cast NativePAGE 3%-12% Bis-Tris gels, while Novex NuPAGE 4%-12% Bis-Tris Gels (Life Technologies) were used for denaturing conditions.

For WB, samples were electrophoretically transferred to PVDF membranes and immunodecorated using commercial specific antibodies. A detailed list can be found in Supplemental Experimental Procedures.

**Immunodetection signal intensities were quantified using ImageJ.**

**Quantitative SILAC Mass Spectrometry**

The two cell lines to be compared by SILAC (Ong et al., 2002) were grown in “heavy” DMEM containing 15N- and 13C-labeled Arg and Lys and in “light” DMEM containing 14N and 12C Arg and Lys (Sigma-Aldrich). Equal portions of the differentially labeled cell lines were mixed and solubilized; affinity purifications were performed using anti-HA-agarose (Cell Signaling), anti-FLAG-agarose (Sigma-Aldrich), or cIV immunocapture kit (Abcam) beads and analyzed as described previously (Andrews et al., 2013). See Supplemental Experimental Procedures for a detailed explanation.

**Complexome Profiling**

Mitochondrial samples from 143B cells and human skin fibroblasts (cell line C-124 from the Radboud Center for Mitochondrial Medicine) or from differentially labeled MT-CO3 mutated and WT cybrids (1:1 mixture of the cells grown in heavy and light SILAC media) were used for “complexome profiling,” which was performed and analyzed as previously described (Heide et al., 2012), with some modifications for SILAC sample analysis. For a detailed explanation, see Supplemental Experimental Procedures.

**Functional Assays**

The activities of COX and citrate synthase (CS) were measured in digitonin-solubilized cell samples (Tiranti et al., 1995) by spectrophotometry as described previously (Kirby et al., 2007). The respiratory activity was measured in whole cells in an XF24 extracellular flux analyzer (Seahorse Biosciences) (Invernizzi et al., 2012). Statistical analysis was by unpaired Student’s t test using GraphPad Prism 5.0a software.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.01.044.

**AUTHOR CONTRIBUTIONS**

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