Oms1 associates with cytochrome c oxidase assembly intermediates to stabilize newly synthesized Cox1

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ABSTRACT  The mitochondrial cytochrome c oxidase assembles in the inner membrane from subunits of dual genetic origin. The assembly process of the enzyme is initiated by membrane insertion of the mitochondria-encoded Cox1 subunit. During complex maturation, transient assembly intermediates, consisting of structural subunits and specialized chaperone-like assembly factors, are formed. In addition, cofactors such as heme and copper have to be inserted into the nascent complex. To regulate the assembly process, the availability of Cox1 is under control of a regulatory feedback cycle in which translation of COX1 mRNA is stalled when assembly intermediates of Cox1 accumulate through inactivation of the translational activator Mss51. Here we isolate a cytochrome c oxidase assembly intermediate in preparatory scale from coa1Δ mutant cells, using Mss51 as bait. We demonstrate that at this stage of assembly, the complex has not yet incorporated the heme a cofactors. Using quantitative mass spectrometry, we define the protein composition of the assembly intermediate and unexpectedly identify the putative methyltransferase Oms1 as a constituent. Our analyses show that Oms1 participates in cytochrome c oxidase assembly by stabilizing newly synthesized Cox1.

INTRODUCTION

The eukaryotic cytochrome c oxidase is a copper–heme a oxidase that acts as the terminal enzyme of the respiratory chain. The enzyme complex is composed of subunits of dual genetic origin. In yeast and humans, Cox1, Cox2, and Cox3 are multispanning inner membrane proteins encoded by the mitochondrial genome that form the core of the complex. These proteins are translated on membrane-associated ribosomes on the matrix side of the inner membrane and have to be inserted into the lipid phase by the mitochondrial protein export machinery. Oxa1 represents the core subunit of this mitochondrial export machinery. The channel-forming Oxa1 protein facilitates the membrane insertion of mitochondrion-encoded proteins in a cotranslational manner (Jia et al., 2003; Szyrach et al., 2003; Krüger et al., 2012). During this process, Oxa1 cooperates with the ribosome associated peripheral membrane protein Mba1 (Ott et al., 2006; Pfeffer et al., 2015). Mba1 is positioned close to the ribosomal exit tunnel and has been suggested to help to align the ribosome with the export machinery (Ott et al., 2006; Bauerschmitt et al., 2010; Pfeffer et al., 2015). A multicopy suppressor screen revealed Oxa1 multicopy suppressor (Oms1; YDR316w) as an Oxa1-related protein that suppresses Oxa1 mutant alleles.
when overexpressed. Of interest, Oms1 contains a predicted C-terminal methyltransferase domain, mutations of which abolish its suppression capabilities (Lemaire et al., 2004). However, the function of Oms1 has remained enigmatic.

The assembly of the cytochrome c oxidase represents a process during which the individual subunits associate in a sequential manner. The assembly process initiates on the membrane-inserted Cox1 protein, which associates transiently with diverse assembly factors and structural subunits to eventually form the active enzyme complex. Several factors in yeast and human mitochondria assist at different steps of the assembly process. Some of these have been functionally linked to distinct assembly intermediates and maturation processes. The identification and characterization of these assembly factors have significantly contributed to our understanding of the assembly process and provided insight into the molecular basis of assembly defects that lead to human disorders caused by cytochrome c oxidase deficiency (Fernández-Vizarra et al., 2009; Mick et al., 2011; Soto et al., 2012; Herrmann et al., 2013).

The assembly of the cytochrome c oxidase has been best analyzed in the yeast Saccharomyces cerevisiae. In yeast, translation of the COX1 mRNA requires the dedicated translational activators Pet309, Pet54, Mam33, and Mss51 (Decoster et al., 1990; Manthey and McEwen, 1995; Barrientos et al., 2002; Pérez-Martínez et al., 2003; Roloff and Henry, 2015; Mayorga et al., 2016). After membrane insertion, the newly synthesized Cox1 associates with the assembly factors Coa3 and Cox14, which stabilize Cox1 (Glerum et al., 1995; Barrientos et al., 2004; Mick et al., 2010; Fontanesi et al., 2011). The Coa3/Cox14/Cox1 complex subsequently engages with the assembly factors Coa1 and Shy1 (Mick et al., 2007; Pierrel et al., 2007). Recruitment of Mss51 to Cox1 assembly intermediates inactivates Mss51, rendering it incompetent to support translation of Cox1. On further maturation of the assembly intermediate, Mss51 dissociates from the nascent cytochrome c oxidase and can stimulate further rounds of COX1 mRNA translation (Barrientos et al., 2002, 2004; Pérez-Martínez et al., 2003; Mick et al., 2007; Pierrel et al., 2007). Of interest, in this process, loss of the assembly factor Coa1 can be suppressed by an increase in the amount of Mss51 and copper supplementation to the growth medium (Pierrel et al., 2004; Mick et al., 2007; Fontanesi et al., 2011).

To confirm the SILAC analysis, we solubilized mitochondria derived from arg4Δcoa1Δmss51Δf strain and performed stable isotope labeling by amino acids in cell culture (SILAC; Ong et al., 2002). To avoid false-positive identifications, we carried out label-switch experiments in which Mss51Δf isolations were performed after switching the medium from heavy to light amino acids between arg4Δcoa1Δmss51Δf and the control strain (Figure 1C). Mass spectrometric analysis revealed selective enrichment of early cytochrome c oxidase–associated factors (Figure 1D and Supplemental Table S1). Of note, the cytochrome c oxidase core subunit Cox1 was the most enriched protein. Furthermore, the previously described cytochrome c oxidase assembly factor Shy1, the structural subunits Cox6 and Cox5, and the mitochondrial chaperone Ssc1 were identified. Peptides of Coa3 and Cox14 were not detected during liquid chromatography–tandem mass spectrometry analysis, probably due to their low molecular weight and hydrophobicity. Most interestingly, Oms1, a protein containing a predicted methyltransferase domain, was significantly enriched with Mss51Δf.

In conclusion, these analyses defined Mss51-interacting proteins. In addition to the early cytochrome c assembly factors and structural subunits, the Oms1 protein was identified as an Mss51-associated protein.

**RESULTS**

**Oms1 is a constituent of COA complexes**

Cytochrome c oxidase assembly progresses through multiple transient assembly intermediates. Owing to their low abundance at steady state, the protein composition of individual assembly intermediates has remained ill defined despite the fact that many of their constituents are known. Previous studies indicated that a lack of the assembly factor Coa1 leads to accumulation of newly synthesized Cox1 in an assembly intermediate (termed COA220 in this study; Khalimonchuk et al., 2010; Mick et al., 2010). This COA220 complex was reminiscent of a previously described Mss51-containing complex (Bareth et al., 2013). On the basis of these observations, we expressed a C-terminally streptavidin- and FLAG-tagged Mss51 (Mss51Δf) in wild-type and coa1Δ backgrounds to purify the COA220 complex. Functionality of this fusion construct was assessed by growth tests of Mss51Δf-expressing cells on fermentable (yeast extract/peptone/glucose [YPD]) and nonfermentable (yeast extract/peptone/glycerol [YPG]) carbon sources. Wild-type cells expressing Mss51Δf from the chromosomal locus displayed wild-type–like growth behavior on both carbon sources (Figure 1A). As expected, coa1Δ cells expressing Mss51Δf failed to grow on nonfermentable medium (Mick et al., 2007; Pierrel et al., 2007). In addition, Mss51Δf-expressing wild-type cells displayed similar mitochondrial protein levels to wild-type mitochondria containing untagged Mss51 (Figure 1B). Thus we concluded that Mss51Δf was functional and could be used for further analyses.

To establish an unbiased identification strategy allowing for a comprehensive identification of proteins present in the COA220 complex, we generated an arg4Δcoa1Δmss51Δf strain and performed stable isotope labeling by amino acids in cell culture (SILAC; Ong et al., 2002). To avoid false-positive identifications, we carried out label-switch experiments in which Mss51Δf isolations were performed after switching the medium from heavy to light amino acids between arg4Δcoa1Δmss51Δf and the control strain (Figure 1C). Mass spectrometric analysis revealed selective enrichment of early cytochrome c oxidase–associated factors (Figure 1D and Supplemental Table S1). Of note, the cytochrome c oxidase core subunit Cox1 was the most enriched protein. Furthermore, the previously described cytochrome c oxidase assembly factor Shy1, the structural subunits Cox6 and Cox5, and the mitochondrial chaperone Ssc1 were identified. Peptides of Coa3 and Cox14 were not detected during liquid chromatography–tandem mass spectrometry analysis, probably due to their low molecular weight and hydrophobicity. Most interestingly, Oms1, a protein containing a predicted methyltransferase domain, was significantly enriched with Mss51Δf.

In conclusion, these analyses defined Mss51-interacting proteins. In addition to the early cytochrome c assembly factors and structural subunits, the Oms1 protein was identified as an Mss51-associated protein.

**COA220 represents a heme-free intermediate**

To confirm the SILAC analysis, we solubilized mitochondria derived from wild-type and Mss51Δf cells, purified Mss51Δf-containing complexes, and analyzed eluates by SDS–PAGE and Western blotting. Mss51Δf was efficiently isolated, as were early cytochrome c oxidase assembly factors (e.g., Shy1), the core subunit Cox1, and early-assembling nuclear-encoded subunits (e.g., Cox5 and Cox6; Figure 2A). Although Coa3 and Cox14 were not enriched in the SILAC mass spectrometric analysis (Figure 1D), we clearly detected them by Western blotting (Figure 2A). To define the Mss51Δf-containing complex in coa1Δ mitochondria, we isolated Mss51Δf under native conditions and analyzed the eluted protein complexes by blue native (BN)-PAGE. Mss51 was solely detected in a complex of 220 kDa (named COA220 together with Cox1, Cox14, and Cox5 (Figure 2B).

Shy1 and its mammalian homologue, SURF1, have been implicated in heme insertion into Cox1 (Bundschuh et al., 2009).
Respiratory chain supercomplexes are altered in oms1Δ mitochondria

The presence of Oms1 in the Mss51SF-isolated COA220 complex, as assessed by mass spectrometry, was unexpected. Oms1 has been described as a mitochondrial protein able to act as an Oxa1 suppressor (Lemaire et al., 2004). To confirm that Oms1 and Mss51 were present in the same complex, we purified Mss51SF from digitonin-solubilized mitochondria. The expected cytochrome c oxidase assembly factors Coa1 and Coa3 coisolated with Mss51. In addition, Oms1 was recovered in the eluate (Figure 3A). To substantiate this finding, we performed the reverse experiment using an antibody directed against the C-terminus of Oms1 for complex isolation. After immunoprecipitation, Mss51 was clearly recovered in the eluate; however, Coa1 could not be detected in the precipitates (Figure 3B). Because Coa1 was identified with Mss51SF but not during immunoprecipitation using an antibody directed against the C-terminus of Oms1, we performed the reverse experiment with an antibody against Coa1. Besides Cox4, Coa3, and Cox1, Oms1 was also detected, suggesting the presence of Mss51, Coa1, and Oms1 in a complex (Figure 3C).
To investigate the function of Oms1, we generated a strain with a chromosomal deletion of OMS1 (oms1Δ) and assessed its growth behavior in comparison to wild-type and coa1Δ cells on full or synthetic media supplemented with fermentable (YPD/synthetic defined medium with glucose [SD]) or nonfermentable (YPG/synthetic defined medium with glycerol [SG]) carbon sources. After incubation at 24, 30, and 37°C, we observed the expected absence of growth of coa1Δ cells (Mick et al., 2007; Pierrel et al., 2007) and a subtle growth defect for oms1Δ cells on nonfermentable full medium (YPG) at 24 and 37°C compared with wild-type cells. The reduction in growth of oms1Δ cells was even more pronounced on synthetic media (Figure 3E). These findings suggested a respiratory chain defect in oms1Δ cells. Hence we analyzed oms1Δ mitochondria with regard to respiratory chain function. The analysis of steady-state protein levels revealed a clear reduction of Cox1 levels, whereas other tested proteins, such as Cox2, Coa1, and Coa3, were only mildly affected (Figure 3F).

To analyze respiratory chain complexes directly, we solubilized mitochondria and separated protein complexes by BN–PAGE. We observed a reduction of the IIIc/IV complex in oms1Δ mitochondria. At the same time, complex IIIc was apparent in mutant mitochondria, whereas other tested proteins, such as Cox2, Coa1, and Coa3, were only mildly affected (Figure 3F). To address whether the activity of respiratory chain complexes was affected, we conducted in-gel activity staining. In agreement with the observed rearrangement of supercomplexes, cytochrome c oxidase activity of the IIIc/IV complex was increased compared with the wild-type sample, whereas reduced activity of the slower-migrating oligomer IIIc/IVc2 was apparent in oms1Δ (Figure 3H). As a control, complex V activity remained unaltered in the mutant sample, confirming the immunoblotting results. As expected from the BN–PAGE analysis (Figure 3G), there was no difference in activity measurements of the cytochrome c reductase in oms1Δ mitochondria compared with wild type (Figure 3I, top). Because the cytochrome c oxidase, as the terminal enzyme of the respiratory chain, reduces molecular oxygen, we performed high-resolution oxygen measurements to follow O2 consumption of wild-type and oms1Δ mitochondria. Oxygen consumption was significantly reduced in oms1Δ mitochondria (Figure 3I, bottom), indicating a respiratory defect. The results taken together show that Oms1 is necessary for proper function of the cytochrome c oxidase and mitochondrial respiration.

To address whether Oms1 was present in mitochondrial respiratory chain supercomplexes, we isolated Cor1ΔΔ containing complexes under native conditions and analyzed copurifying proteins by Western blotting. In addition to the expected complex III and IV subunits, we recovered assembly factors (e.g., Cox14) and supercomplex-associated factors (e.g., Rcf2; Chen et al., 2012; Strogolova et al., 2012; Vukotic et al., 2012). However, Oms1 was not identified in detectable amounts (Figure 3D). Thus we hypothesized that Oms1 is present only in the COA220 complex.
Mutations in the methyltransferase domain of Oms1 do not affect cytochrome c oxidase function

Oms1 contains a predicted methyltransferase domain that is critical for its functions as an Oxa1 suppressor (Lemaire et al., 2004). This finding prompted us to investigate the function of the putative methyltransferase domain with regard to the observed cytochrome c oxidase defect. We used site-directed mutagenesis to create plasmids carrying the endogenous promoter and the OMS1 open reading frame with the previously described methyltransferase-domain mutations (Lemaire et al., 2004) as either single or combined mutations. In addition, we investigated effects of Oms1 overexpression (wild-type and mutant forms). The oms1Δ cells were either transfected with centromeric (pRS416) or high-copy (pRS426) plasmids carrying the different constructs. Because the growth defect of oms1Δ cells was most pronounced on synthetic media, we investigated cell growth on SD and SG media at various temperatures. As a control, we used cox15Δ cells transformed with the empty pRS416 plasmid. Although we observed the described growth phenotype of oms1Δ, all tested mutations were able to restore cell growth (Figure 4A). Subsequently, we isolated mitochondria from oms1Δ cells containing the empty plasmid or the wild-type (WT) OMS1-containing plasmid or expressing the double mutant of OMS1 (encoding Oms1\textsuperscript{DT}\textsuperscript{AA}). Because deletion of OMS1 led to an altered respiratory chain complex organization, we solubilized oms1Δ mitochondria containing an empty plasmid or the plasmid-encoded Oms1 variants in digitonin buffer and subjected the extracts to BN–PAGE and Western blotting. Expression of Oms1\textsuperscript{WT} restored supercomplex formation in mitochondria. Surprisingly, the Oms1\textsuperscript{DT}\textsuperscript{AA} protein also restored supercomplex formation to WT levels (Figure 4B). In addition, we performed oxygen consumption measurements of mitochondria from oms1Δ cells and cells expressing Oms1\textsuperscript{WT} and Oms1\textsuperscript{DT}\textsuperscript{AA}. Whereas oxygen consumption in oms1Δ mitochondria was reproducibly decreased, Oms1\textsuperscript{WT} and and oms1Δ (gray bars) mitochondria (n = 3, ±SEM). Bottom, oxygen consumption measurement of mitochondria from WT (white bars) and oms1Δ (gray bars). Isolated mitochondria were incubated with NADH (basal) and successively combined with ADP (phosphorylating respiration; state 3) in a high-resolution respirometry chamber (n = 4, ±SEM).
Oms1 stabilizes newly synthesized Cox1

We identified Oms1 as a protein copurifying with Mss51 under conditions of an accumulating early cytochrome c oxidase assembly intermediate. Loss of Oms1 leads to supercomplex rearrangements, reduced Cox1 steady-state levels, and reduced cytochrome c oxidase activity. Because assembly intermediates are short-lived under physiological conditions and of low abundance in mitochondria, we speculated that an interaction of Oms1 with COA220 was likely transient in wild-type cells. To support this hypothesis, we carried out Oms1 immunoprecipitation analyses from mitochondrial extracts obtained from wild-type and coa1Δ mutant mitochondria with accumulated COA220 complex. Oms1 was efficiently isolated from both extracts (Figure 5A). As expected, Mss51 and Coa3 were enriched in samples obtained from coa1Δ mitochondria, whereas a small amount of Mss51 was recovered in the wild-type sample at steady state (see also Figure 3B). Thus Mss51 coimmunoprecipitation efficiency correlated with the abundance of the COA220 intermediate. To investigate further the interaction of Mss51 and Oms1 in early cytochrome c oxidase assembly steps, we performed Oms1 immunoprecipitations from coa3Δ, cox14Δ, and cox1− mitochondrial extracts. Mss51 could be detected only in the wild-type sample (Figure 5B). This observation suggests that Oms1 association with Mss51 depends on the presence of Cox1 and the formation of Cox1-containing assembly intermediates.

Because Mss51 is required for COX1 mRNA translation and thus Cox1 expression, we analyzed whether lack of Oms1 affected Cox1 synthesis or stability. To investigate Cox1 synthesis in oms1Δ cells,
we performed in vivo pulse labeling experiments. However, we did not observe any changes in mitochondrial protein synthesis in oms1Δ mitochondria (Figure 5C). Accordingly, the observed reduction in Cox1 that we observe in oms1Δ cells could not be attributed to defect in its synthesis. Alternatively, the reduced amount of Cox1 could be due to an increased turnover of Cox1. To address this, we pulse labeled mitochondrial translation products and analyzed labeled products after an extended chase. A significantly increased turnover rate of Cox1 was apparent in oms1Δ compared with wild-type cells and to a lesser extent also for Cox2 (Figure 5D). A quantitative analysis showed a degradation of Cox1 to 45% after a 5-min chase compared with the wild-type control but no further decrease during an extended chase of 45 min (Figure 5E). In addition, newly synthesized Cox2 displayed an increased turnover to 73% (after a 5-min chase) and to 58% (after 45 min). In contrast, Cob stability was not affected in oms1Δ cells. In conclusion, Oms1 is a transient interactor of Cox1 and present in the COA220 complex. Although it is not required for Cox1 translation, Oms1 assists in stabilizing Cox1 during early steps of cytochrome c oxidase assembly. Because no protein involved in Cox2 maturation was found in the SILAC analysis together with Oms1 and Mss51 and previous reports also described destabilization of Cox2 when Cox1 is lacking (Barrientos et al., 2002; Pérez-Martínez et al., 2003; Mick et al., 2007, 2010; Pierrel et al., 2007, 2008; Fontanesi et al., 2011), the decreased stability of Cox2 is probably indirect.

Lemaire et al. (2004) suggested that Oms1 might methylate Oxa1, rendering it resistant to Oma1 protease-mediated turnover, and hence support Cox1 biogenesis indirectly. Moreover, Bestwick et al. (2010) found in coa2Δ cells that instability of Cox1 could be overcome by deletion of OMA1. Hence, to test a role of Oma1 in the turnover of Cox1, we generated a chromosomal deletion of OMA1 (oma1Δ) and a double mutant strain lacking OMS1 and OMA1 (oms1Δ/oma1Δ). We assessed the growth media. Yeast cells from the indicated strains were spotted in serial 10-fold dilutions and incubated at 30°C. (G) Quantification of in vivo labeling of mitochondrial translational products. Cells were labeled for 5 min and chased for 15 min. TCA-precipitated samples were separated by SDS–PAGE, quantified by digital autoradiography, and normalized for Var1 (C). n = 3; ± SEM.
behavior of these strains on full or synthetic media containing fermentable (YPD/SD) or nonfermentable (YPG/SG) carbon sources. We used WT and coa1Δ cells as control. We reproducibly observed the growth defect of oms1Δ cells on nonfermentable media (Figure 5F). Whereas the single-mutant oma1Δ strain showed no growth defect, oms1Δ/oma1Δ cells displayed the same growth phenotype as oms1Δ cells (Figure 5F). To investigate Cox1 stability directly, we performed in vivo pulse labeling of mitochondrial translation products in oms1Δ and oms1Δ/oma1Δ cells in comparison to oma1Δ. Whereas Cox1 stability was significantly reduced in oms1Δ cells, we observed a subtle increase of Cox1 stability in the double oms1Δ/oma1Δ strain (Figure 5G). These analyses indicate that Oma1 contributes to the turnover of Cox1 in oms1Δ mutant mitochondria. However, in addition to Oma1, other proteases appear to be involved in this process.

**DISCUSSION**

Assembly of the cytochrome c oxidase initiates with the synthesis of Cox1. After cotranslational insertion of the newly synthesized Cox1 into the inner mitochondrial membrane, the protein becomes stabilized through interactions with assembly factors such as Cox14 and Coa3 (Barrientos et al., 2004; Pérez-Martínez et al., 2009; Mick et al., 2010; Fontanesi et al., 2011). Current concepts on the assembly process suggest that in successive steps additional assembly factors associate, cofactors are inserted into Cox1, and nuclear- and mitochondria-encoded subunits are recruited (Taannam and Williams, 2001; Herrmann and Funes, 2005; Mick et al., 2011; Fox, 2012; Soto et al., 2012; Richter-Dennerlein et al., 2015). Because assembly factors accompany Cox1 during successive stages of the assembly process, individual assembly intermediates are difficult to purify and therefore have not been defined biochemically. Here we used a coa1Δ mutant in which newly synthesized Cox1 forms a stable intermediate to isolate and dissect this complex (Khalimonchuk et al., 2010; Mick et al., 2010). Our analyses indicate that the purified COA220 complex has not yet received the heme cofactors. In addition, we identified Oms1 as an unexpected constituent of the complex. Our analyses indicate that Oms1 is a transient interactor of the assembly pipeline, since we find only a significant interaction with assembly intermediates under conditions of an accumulating intermediate. It is interesting that Oms1 was linked previously to respiratory chain biogenesis, as it was identified as a suppressor of OX1 mutant alleles. In that case, the methyltransferase domain of Oms1 was shown to be critical for the suppression phenotype (Lemaire et al., 2004). However, with regard to the oms1Δ growth defect that we observed and the underlying cytochrome c oxidase defect, we show that a functional methyltransferase domain is dispensable. These findings suggest that two distinct activities can be attributed to Oms1.

Although the molecular basis for the observed genetic interaction with Oxa1 remains unexplained, a lack of Oms1 affects the stability of newly synthesized Cox1. However, despite this defect, a significant amount of Cox1 is apparently maintained in an assembly-competent state. The reduction of Cox1 seen at steady state in oms1Δ mitochondria is much less pronounced than what is seen in cox1Δ or coa3Δ. Hence lack of Cox14 and Coa3 destabilizes Cox1, but at the same time, both proteins interact with the translational activator Mss51 and Cox1 early during the assembly process (Barrientos et al., 2004; Pérez-Martínez et al., 2009; Mick et al., 2010; Fontanesi et al., 2011). Our analyses indicate that although the association of Oms1 with COA220 appears to be transient and much less apparent at steady state, an association of Oms1 with Mss51 can be detected when Coa1 is lacking. This finding suggests that Oms1 is primarily engaged with Mss51 and likely recruited to the COA220 complex by Mss51. However, given the role of Mss51 in translation of Cox1, this hypothesis cannot easily be assessed experimentally. Moreover, it will be important to address whether Oms1 acts directly on Cox1 or affects stability indirectly via Cox14, Coa3, or another constituent of the COA220 intermediate.

**MATERIALS AND METHODS**

**Yeast strains, molecular cloning, and mitochondrial preparation**

All S. cerevisiae strains, used in this study with the exception of cox1Δ, are listed in Table 1. Chromosomal deletions of COA1, ARG4, OMA1, COA3, COX14, and OMS1, double-mutant strains, and tagged versions of Mss51 were generated by introduction of the TRP1, NatNT2, or His3MX6 cassettes using PCR-based strategies (Knop et al., 1999; Janke et al., 2004). The cox1Δ yeast cells were cultured as described previously (Mick et al., 2010). Streptavidin-FLAG–tagged Mss51 (Mss51Δ59) was generated using a modified pYMY2.1 vector (Alkhaja et al., 2012). OMS1 was cloned with its endogenous promoter and terminator from yeast (YPH499) genomic DNA into pRS416 and pRS426 (Stratagene). Point mutations were introduced by site-directed mutagenesis (QuickChange; Stratagene, Cambridge, United Kingdom) according to the manufacturer’s specifications. Yeast strains were transformed using the lithium acetate method and confirmed by PCR or Western blot analysis. Yeast cells were grown in liquid medium containing 1% yeast extract, 2% peptone and 2% glucose, 1% galactose, or 3% glycerol (YPD/YPGal/YPG, respectively). Strains containing plasmid-borne wild-type or mutant form of OMS1 were grown on synthetic medium (0.67% yeast nitrogen base, 0.07% complete supplement mixture –Ura) and 1% galactose. If not indicated otherwise, yeast cells were grown at 30°C. For growth tests, liquid precultures were adjusted to OD600 0.3, and serial 1:10 dilutions were spotted on solid media plates and incubated for 3–5 d at the indicated temperatures. Mitochondria were isolated essentially as previously described (Meisinger et al., 2006). For steady-state analysis of mitochondrial proteins, different amounts of mitochondria were subjected to SDS–PAGE, followed by Western blot analysis.

**Native protein complex isolation**

Mitochondria were solubilized in 20 mM Tris/HCl (pH 7.4), 100 mM NaCl, 10% (wt/vol) glycerol, 5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 1% digitonin for 30 min at 4°C. The solubilized material was cleared (20,000 × g, 15 min, 4°C) and the mitochondrial extract applied to the respective resin for 1–2 h at 4°C. After extensive washing (20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 10% (wt/vol) glycerol, 5 mM EDTA, 2 mM PMSF, 0.3% digitonin), bound material was eluted, mixed with the appropriate loading dye and analyzed by SDS- or BN–PAGE. For immunoglobulin G (IgG) chromatography of Cox420S and Cox1R65 human IgGs (Sigma-Aldrich, St. Louis, MO) were coupled to CNBr-activated Sepharose (GE Healthcare, Little Chalfont, United Kingdom) according to the manufacturer’s specifications. Bound material was eluted by tobacco etch virus (TEV) protease (Invitrogen, Carlsbad, CA) treatment. TEV protease carrying a polyhistidine tag was removed by addition of Ni–nitrilotriacetic acid (Rehling et al., 2003).

Co-immunoprecipitation was performed as described (Hutu et al., 2008; Mick et al., 2010). Coa1- and Oms1-specific antisera were bound to Protein A–Sepharose (GE Healthcare) in 0.1 M potassium phosphate buffer (pH 7.4) for 1 h at room temperature and subsequently cross-linked with 5 mg/ml dimethyl pimelimidate (DMP) in 0.1 M sodium borate (pH 9.0) for 30 min at room temperature. DMP
was quenched with 1 M Tris/HCl (pH 7.4). After binding and washing, proteins were eluted with 0.1 M glycine (pH 2.8) and immediately neutralized with 1 M Tris (pH 11.5). For isolation of Mss51<sup>159</sup>, solubilized mitochondria were bound to preequilibrated Strep-Tactin Sepharose (IBA, Göttingen, Germany) for 1 h at 4°C. Bound protein complexes were eluted with elution buffer (5 mM desthiobiotin in digitonin wash buffer) for 15 min at 12°C for native analysis or with 0.1 M glycine (pH 2.8). For mass spectrometric (MS) analysis, samples were trichloroacetic acid (TCA) precipitated and resuspended in 20 mM Tris/HCl (pH 7.4), 100 mM NaCl, 10% (wt/vol) glycerol, and 5 mM EDTA.

**TABLE 1: Yeast strains used in this study.**

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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<tr>
<td>coa1Δ Mss51&lt;sup&gt;159&lt;/sup&gt;Flag arg4Δ (BBY13)</td>
<td>Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; coa1::klTRP1; mss51::mss51-Strap-FLAG-His3MX6; arg4::natNT2</td>
<td>This study</td>
</tr>
<tr>
<td>Cor1&lt;sup&gt;TAP&lt;/sup&gt;</td>
<td>Mat a, his3-Δ1, leu2Δ0, met15Δ0, ura3Δ0; cor1::cor1-TAP</td>
<td>Vukotic et al., 2012</td>
</tr>
<tr>
<td>Cox4&lt;sup&gt;ProtA&lt;/sup&gt;</td>
<td>Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; cox4::cox4-TEV-Prota-7His-His3MX6</td>
<td>Vukotic et al., 2012</td>
</tr>
<tr>
<td>oms1Δ (BBY28)</td>
<td>Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; oms1::klTRP1</td>
<td>This study</td>
</tr>
<tr>
<td>oms1Δ oms1Δ (932)</td>
<td>Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; oms1::klTRP1; oms1::His3MX6</td>
<td>This study</td>
</tr>
<tr>
<td>oms1Δ + pRS416 (BBY77)</td>
<td>Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; oms1::klTRP1 [pRS416]</td>
<td>This study</td>
</tr>
<tr>
<td>oms1Δ + Oms1&lt;sup&gt; WT&lt;/sup&gt; (BBY69)</td>
<td>Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; oms1::klTRP1 [pRS416-OMS1]</td>
<td>This study</td>
</tr>
<tr>
<td>oms1Δ + Oms1&lt;sup&gt;D1326A&lt;/sup&gt; (BBY70)</td>
<td>Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; oms1::klTRP1 [pRS416-OMS1&lt;sup&gt;D1326A&lt;/sup&gt;]</td>
<td>This study</td>
</tr>
<tr>
<td>oms1Δ + Oms1&lt;sup&gt;T327A&lt;/sup&gt; (BBY71)</td>
<td>Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; oms1::klTRP1 [pRS416-OMS1&lt;sup&gt;T327A&lt;/sup&gt;]</td>
<td>This study</td>
</tr>
<tr>
<td>oms1Δ + Oms1&lt;sup&gt;D1326A&lt;/sup&gt; (BBY72)</td>
<td>Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; oms1::klTRP1 [pRS416-OMS1&lt;sup&gt;D1326A&lt;/sup&gt;]</td>
<td>This study</td>
</tr>
<tr>
<td>oms1Δ + Oms1&lt;sup&gt;D1326A&lt;/sup&gt; (2µ) (BBY74)</td>
<td>Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; oms1::klTRP1 [pRS426-OMS1&lt;sup&gt;D1326A&lt;/sup&gt;]</td>
<td>This study</td>
</tr>
<tr>
<td>oms1Δ + Oms1&lt;sup&gt;T327A&lt;/sup&gt; (2µ) (BBY75)</td>
<td>Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; oms1::klTRP1 [pRS426-OMS1&lt;sup&gt;T327A&lt;/sup&gt;]</td>
<td>This study</td>
</tr>
<tr>
<td>oms1Δ + Oms1&lt;sup&gt;D1326A&lt;/sup&gt; (2µ) (BBY76)</td>
<td>Mat a, ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801; oms1::klTRP1 [pRS426-OMS1&lt;sup&gt;D1326A&lt;/sup&gt;]</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Mass spectrometry and SILAC**

For SILAC, yeast strains auxotroph for lysine and arginine were used (coa1<sup>Δarg4Δ</sup>/coa1<sup>Δarg4Δ</sup>Mss51<sup>159</sup>). Both strains were grown in medium containing heavy amino acids and medium containing natural (light) amino acids. Yeast cells were grown in SILAC medium (0.67% yeast nitrogen base, 20 mg/l histidine, 20 mg/l tryptophan, 20 mg/l adenine, 20 mg/l methionine, 20 mg/l uracil, 30 mg/l isoleucine, 30 mg/l tyrosine, 50 mg/l phenylalanine, 100 mg/ml leucine, 150 mg/l valine, 200 mg/l threonine, 200 mg/l proline, 20 mg/l [heavy or light, respectively] lysine, and 20 mg/l [heavy or light, respectively] arginine) supplied with 2% galactose.
at 30°C. Proline (200 mg/l) was used to prevent the conversion of arginine into proline. Mitochondrial preparation followed the procedure described. Before complex isolation, mitochondria from cells grown on light and heavy media were mixed in a 1:1 ratio and solubilized together. For SILAC interactome analysis, eluted proteins were separated on 4–12% gradient SDS–PAGE gels (Invitrogen) and stained with colloidal Coomassie blue. Each gel lane was cut into 23 equal slices, and proteins therein were in-gel digested with trypsin (Promega; Shevchenko et al., 2006). Tryptic peptides from each gel slice were analyzed by nanoflow HPLC coupled to nancoelectrospray LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) under standard conditions. Raw MS data were analyzed with MaxQuant and Andromeda (version 1.2.2.5; Cox and Mann, 2008; Cox et al., 2011) using the S. cerevisiae UniProt reference protein database (version 29.11.11; 64,016 entries). Default double SILAC settings of MaxQuant were used with carbamidomethylation of cysteins and oxidation of methionines set as variable modifications. Results from MaxQuant were analyzed and visualized with R as described (Nikolov et al., 2011).

**Extraction and HPLC analysis of hemes in cytochrome c oxidase**

Complexes containing CoxA or Mss51 were purified from digitonin solubilized mitochondrial lysates using IgG-Sepharose or StrepTactin agarose, respectively. Hemes were extracted from purified complexes and analyzed as described previously (Hildenbeutel et al., 2014). Briefly, samples were treated with 0.14% HCl in acetone, clarified by centrifugation, and analyzed by reverse-phase HPLC. To this end, extracts were loaded at 0.5 ml/min onto a 150-mm YMC ODS-A column (5 µm, 300 Å) at a Shimadzu Scientific HPLC Instrument in 75% buffer A (0.1% trifluoroacetic acid [TFA]/H₂O) and 25% buffer B (0.1% TFA/CH₃CN). Hemes were resolved using a 1%/min gradient from 55–75% buffer B and detected by ultraviolet/visible spectroscopy at 400 nm.

**BN–PAGE and in-gel activity staining**

Mitochondria were solubilized in 1% digitonin, 20 mM Tris/HCl (pH 7.4), 5 mM EDTA, 100 mM NaCl, 10% (wt/vol) glycerol, and 2 mM PMSF to a final concentration of 1 mg/ml for 30 min at 4°C. Lysates were cleared by centrifugation (20,000 × g, 15 min, 4°C) before addition of 10x loading dye (5% Coomassie brilliant blue G-250, 500 mM 6-aminohexanoic acid, 100 mM Bis-Tris, pH 7.0) and separated on 4–13% polyacrylamide gradient gels with 4% stacking gel as described (Wittig et al., 2007). Activity staining of respiratory chain complexes was performed at 30°C according to published procedures (Wittig et al., 2007; Deckers et al., 2014). For complex IV staining, gel stripes were incubated in 50 mM KPi (pH 7.4), 0.5 mg/ml diaminobenzidine, and 1 mg/ml reduced cytochrome c. Complex V staining was performed in 35 mM Tris/HCl, 220 mM glycine (pH 8.3), 8 mM ATP, 14 mM MgSO₄, and 0.2% Pb(NO₃)₂.

**In vivo labeling of mitochondrial translational products**

In vivo labeling was performed in whole cells (grown on YPGal) in 40 mM KPi (pH 6.0), 2% galactose, 150 µg/ml cycloheximide, and 20 µCi of [³⁵S]methionine as described previously (Mick et al., 2010). After 10 min (for pulse-only experiments) or 5 min (for pulse-chase experiments) of labeling at 30°C, reactions were stopped by addition of 10 mM unlabeled methionine. For chase experiments, samples were further incubated for the indicated times at 30°C, and proteins were extracted by alkaline treatment, precipitated with 10% TCA, and analyzed by SDS–PAGE and digital autoradiography.

**Isolated enzyme activity measurement**

Spectrophotometric analysis of isolated respiratory chain complex activities was done using a Cary 50 Bio UV/Vis spectrophotometer as previously described (Deckers et al., 2014). Cytochrome c reductase (CIII) activity was determined by the change of absorbance at 550 nm during reduction of cytochrome c. Mitochondria were added to sample buffer (40 mM potassium phosphate buffer, pH 7.4, 0.5 mM NADH) containing 0.02% (wt/vol) oxidized cytochrome c.

**Determination of mitochondrial respiration**

Mitochondrial oxygen consumption rate was measured essentially as previously described with minor changes (Mourier et al., 2014). Isolated yeast mitochondria (10 mg) were diluted in 2 ml of mitochondrial respiration buffer (120 mM sucrose, 220 mM mannitol, 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/HCl, 10 mM KH₂PO₄, 5 mM MgCl₂, 1 mM EGTA, pH 7.4) in an Oxygraph-2k (OROBOROS Instruments, Innsbruck, Austria). The oxygen consumption rate was measured using 1 mM NADH at 30°C. Oxygen consumption was assessed in the basal state and in the phosphorylating state with 1 mM ADP (state 3).

**Additional procedures**

Standard techniques were applied for SDS–PAGE and Western blotting onto polyvinyldiene fluoride membranes. All primary antibodies used were raised in rabbits. For detection and visualization of antibody–protein complexes on x-ray films, peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) and enhanced chemiluminescence reagent (GE Healthcare) were used. Radioactive proteins were analyzed by digital autoradiography, and signals were quantified with the Image Quant software (GE Healthcare).

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