Respiratory chain supercomplexes associate with the cysteine desulphurase complex of the iron–sulfur cluster assembly machinery

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ABSTRACT Mitochondria are the powerhouses of eukaryotic cells. The activity of the respiratory chain complexes generates a proton gradient across the inner membrane, which is used by the F₁F₉-ATP synthase to produce ATP for cellular metabolism. In baker’s yeast, Saccharomyces cerevisiae, the cytochrome bc₁ complex (complex III) and cytochrome c oxidase (complex IV) associate in respiratory chain supercomplexes. Iron–sulfur clusters (ISC) form reactive centers of respiratory chain complexes. The assembly of ISC occurs in the mitochondrial matrix and is essential for cell viability. The cysteine desulphurase Nfs₁ provides sulfur for ISC assembly and forms with partner proteins the ISC-biogenesis desulphurase complex (ISD complex). Here, we report an unexpected interaction of the active ISD complex with the cytochrome bc₁ complex and cytochrome c oxidase. The individual deletion of complex III or complex IV blocks the association of the ISD complex with respiratory chain components. We conclude that the ISD complex binds selectively to respiratory chain supercomplexes. We propose that this molecular link contributes to coordination of iron–sulfur cluster formation with respiratory activity.

INTRODUCTION Mitochondria fulfill various central functions for the survival of eukaryotic cells, including biosynthesis of lipids, amino acids, heme, the assembly of iron–sulfur clusters (ISC), and apoptotic signaling. They are referred to as the powerhouses of the cell, since they produce the large majority of cellular ATP via oxidative phosphorylation. Respiratory chain complexes transfer electrons from reducing equivalents onto oxygen to produce water and pump protons across the inner membrane. Thereby, a proton gradient is established, which drives the F₁F₉-ATP synthase to generate ATP from ADP and phosphate (Saraste, 1999; Wallace, 2012; Milenkovic et al., 2017).

In baker’s yeast, Saccharomyces cerevisiae, the respiratory chain is composed of three protein complexes: succinate dehydrogenase (complex II), cytochrome bc₁ complex (complex III), and cytochrome c oxidase (complex IV). The proton-pumping cytochrome bc₁ complex and cytochrome c oxidase interact in respiratory chain supercomplexes (Cruciat et al., 2000; Schägger and Pfeiffer, 2000; Zara et al., 2007; Böttinger et al., 2012). Both complexes are composed of several proteins of dual genetic origin. The mitochondrial genome encodes for one complex III and three complex IV subunits, which form the reactive cores of these protein machineries. In contrast, all other subunits are encoded in the nucleus, synthesized on cytosolic ribosomes, and imported into mitochondria by dedicated protein machineries (Endo et al., 2011; Hewitt et al., 2011;
RESULTS

Respiratory chain complex III and complex IV associate with Nfs1 and Isd11

To identify novel binding partners of the cytochrome c oxidase, we performed affinity purification via His-tagged Cox4. Cox4 is a matrix-localized subunit that is critical for the formation of mature complex IV (Frazier et al., 2006; Coyne et al., 2007; Böttinger et al., 2013). We have established that Hsp70 of Cox4 does not interfere with the stability of complex IV (Böttinger et al., 2013). Mitochondria were isolated from cells grown under respiratory conditions, solubilized with the nonionic detergent digitonin, and incubated with Ni-NTA agarose. Surprisingly, a fraction of the cytochrome desulfurase Nfs1 and its binding partner Isd11 of the ISC assembly machinery were efficiently copurified along with Cox4 (Figure 1A, lane 4). As expected, we found also the subunits Cox1, Cox2, and Cox9 of the cytochrome c oxidase as well as known interaction partners like the mitochondrial (mt) Hsp70, the nucleotide-exchange factor Mge1, and the J-protein Pat1 in the Cox4 elution (Figure 1A, lanes 4 and 8) (Wiedemann et al., 2007; Böttinger et al., 2013). Other control proteins from the mitochondrial matrix, inner and outer membranes were not coeluted (Figure 1A, lane 8), revealing the specificity of the observed association of Nfs1 and Isd11 with Cox4. Interestingly, Nfs1 also binds to Cox4 when cells were grown under fermentative conditions with sucrose as carbon source (YPS) (Figure 1B, left panel, lane 4). However, the amount of copurified Nfs1 via Cox4 was reduced in comparison to nonfermentative growth conditions (YPG) (Figure 1B, right panel). Cox4 is present in at least two populations. The majority of Cox4 is assembled into the mature cytochrome c oxidase, while a smaller fraction robustly associates with mHsp70 (Böttinger et al., 2013). We asked whether the interaction of Cox4 with Nfs1/Isd11 occurs in the context of the mature complex IV or at the mHsp70-bound pool. To address this issue, we expressed His-tagged Cox4 in the mss51 deletion background. Mss51 is a translational activator of the mitochondria-encoded Cox1 (Decoster et al., 1990; Perez-Martinez et al., 2003; Barrientos et al., 2004). In the absence of Mss51 levels of Cox1 are strongly diminished and mature complex IV is virtually absent (Barrientos et al., 2004; Soto et al., 2012b; Böttinger et al., 2013). In our affinity purification, we found that Nfs1 and Isd11 like components of complex III (Rip1, Cin1) do not interact with Cox4 in the mss51 deletion mitochondria (Figure 1C, lane 6). In contrast, binding of mHsp70 to Cox4 is not affected (Figure 1C, lane 6) as reported (Böttinger et al., 2013). We conclude that the copurification of Nfs1 and Isd11 to Cox4 depends on the presence of mature complex IV.

We asked whether Nfs1 and Isd11 also bind to complex III of the respiratory chain that contains a bound ISC. Therefore, we performed affinity purification via TAP-tagged Cor1 (van der Laan et al., 2006), which is a central subunit of complex III. Indeed, Nfs1, Isd11, and components of complex IV (Cox4 and Cox6) were efficiently copurified along with Cor1 in the Cor1TAP elution (Figure 2A, lanes 4 and 8). Quantification reveals that a similar fraction of Nfs1 was bound to Cor1TAP and Cox4 in the Cor1TAP elution (Figure 1B). As control, Tim23 was copurified along with Cor1TAP as reported (van der Laan et al., 2006), while other matrix proteins were not coeluted (Figure 2A, lane 8). We wondered whether Nfs1 and Isd11 associate with the succinate dehydrogenase (complex II) as well. To address this point, we performed affinity purification via Protein A–tagged Sdh4. The complex II subunits Sdh1 and Sdh2 were efficiently copurified via Protein A–tagged Sdh4, indicating that the affinity tag does not disturb complex integrity (Figure 2B, lane 4) (Gebert et al., 2011). In contrast, we did not detect Nfs1, Isd11, Aco1 or subunits of complex III (Rip1, Cyx1) and complex IV (Cox1, Cox2, Cox4) in the elution fraction of the affinity purification via Protein A–tagged Sdh4 (Figure 2B, lanes 4 and 8). Thus, we conclude that a fraction of Nfs1 and Isd11 specifically associates with complex III and complex IV but not with the succinate dehydrogenase of the respiratory chain.
To confirm the specific binding of ISD components to respiratory chain complexes III and IV, we aimed to perform reverse pull-down assays via Nfs1. To this end, we generated a yeast strain that expresses His-tagged Nfs1. The mutated yeast cells grew on a nonfermentable carbon source, indicating that the function of the essential Nfs1 is not compromised by the fused His tag. Furthermore, in affinity purifications from lysed mitochondrial extract, Isd11 efficiently bound to His-tagged Nfs1, confirming the integrity of the cysteine desulfurase complex (Figure 3A, lane 4). Strikingly, small amounts of complex III (Rip1) and complex IV (Cox1, Cox2, Cox9) subunits were copurified along with His-tagged Nfs1, while various matrix proteins and complex II subunits were not coeluted with Nfs1His (Figure 3A, lanes 4 and 8). To rule out the possibility that tagging of proteins lead to artificial protein–protein interaction, we used a coimmunoprecipitation approach with Nfs1-specific antibodies coupled to Protein A Sepharose (Figure 3B). Nfs1 and Isd11 were efficiently precipitated with Nfs1 antibodies but not with antibodies of the corresponding preimmune serum (Figure 3B, lanes 3 and 4). Components of the cytochrome bc1 complex (Rip1 and Qcr8) and cytochrome c oxidase (Cox1) were found in the elution fraction, while Atp4 of the inner membrane and Mdh1 of the mitochondrial matrix were not copurified (Figure 3B, lane 4). We analyzed whether other factors of the ISC assembly machinery associate with respiratory chain complexes. Recently, Acp1 was identified as novel subunit of the ISD complex (van Vranken et al., 2016; Boniecki et al., 2017; Cory et al., 2017). We generated a yeast strain that expresses His-tagged Acp1 and performed affinity purification from lysed mitochondria. Nfs1 was efficiently copurified along with Acp1His, revealing that the integrity of the ISD complex is intact. Similarly to our affinity purifications via Nfs1His, several subunits of complex III (Qcr8, Rip1) and complex IV (Cox1, Cox2) of the respiratory chain were co-eluted with Acp1His, whereas subunits of complex II (Sdh1, Sdh4) and several matrix proteins were not present in the Acp1His-bound fraction (Figure 3C, lanes 4 and 8). In contrast, no detectable amounts of respiratory chain subunits bound to His-tagged Isu1 (Figure 3D, lane 4) or His-tagged Yfh1 (Figure 3E, lane 4). Nfs1, a known transient interaction partner of Isu1 and Yfh1 (Gerber et al., 2003; Wiedemann et al., 2006; Webert et al., 2014), was present in both elution fractions (Figure 3, D and E, lane 4). Thus, we conclude that Nfs1, Isd11, and Acp1 bind specifically to respiratory chain complexes III and IV.

**Respiratory chain complexes bind to the active cysteine desulfurase complex**

We wondered whether association of Nfs1 to respiratory chain complexes depends on its association with the partner protein Isd11. Therefore, we utilized the temperature-sensitive mutant strain isd11-1, in which the activity of the ISD complex is strongly affected (Wiedemann et al., 2006). To minimize secondary effects on respiratory chain complexes and prevent aggregation of Nfs1, cells were

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**FIGURE 1:** Respiratory chain complex IV associates with Nfs1 and Isd11. (A) Wild-type and Cox4His cells were grown under nonfermentative conditions with glycerol (YPG) as a carbon source. Isolated mitochondria were lysed with digitonin and incubated with Ni-NTA agarose. Load (3%) and elution fractions (100%) were analyzed by SDS–PAGE followed by immunodetection with the indicated antisera. (B) Left panel, wild-type and Cox4His cells were grown under fermentative conditions with sucrose (YPS) as carbon source. Isolated mitochondria were lysed with digitonin and incubated with Ni-NTA agarose. Load (3%) and elution fractions (100%) were analyzed by SDS–PAGE followed by immunodetection with the indicated antisera. Right panel, quantification of the copurification of Nfs1 via Cor1TAP and Cox4His from cells grown under respiratory (YPG) and fermentative growth conditions (YPS). Depicted are mean values of four (Cor1TAP YPG), five (Cox4His YPG), and four (Cox4His YPS) independent experiments as shown in A and B and Figure 2A with the corresponding SEM. (C) Wild-type, Cox4His, and Cox4His mss51Δ mitochondria were lysed with digitonin and incubated with Ni-NTA agarose. Load (1%) and elution fractions (100%) were analyzed by SDS–PAGE followed by immunodetection with the indicated antisera.
Nfs1, Isd11, and Acp1 form a stable complex that can be detected by blue native electrophoresis (Adam et al., 2006; Wiedemann et al., 2006; van Vranken et al., 2016). We asked whether the respiratory chain-bound Nfs1, Isd11, and Acp1 are part of an assembled and active ISD complex. Therefore, we analyzed the affinity purification via Cox4\textsubscript{His} by blue native electrophoresis and indeed detected the ISD complex of ~230 kDa in the elution sample with antibodies against Nfs1 (Figure 5A, lane 4). Since we do not have antibodies that allow detection of Isd11 and Acp1 in the ISD complex on blue native gels, we used a different approach to reveal the presence of these proteins in the Cox4\textsubscript{His}-bound complex. We synthesized \textsuperscript{35}S-labeled Isd11 and Acp1 in a cell-free translation system and individually imported the proteins into isolated Cox4\textsubscript{His}-mitochondria. Imported Isd11 and Acp1 efficiently assembled into the ISD complex of 230 kDa (Figure 5B, lanes 1, 2, 5, and 6) (Wiedemann et al., 2006; van Vranken et al., 2016), which could be copurified along with Cox4\textsubscript{His} (Figure 5B, lanes 4 and 8). Thus, assembled ISD complex associates with respiratory chain complexes. To check whether the respiratory chain-associated ISD complex is active, we took advantage of the desulfurase activity of Nfs1. After releasing sulfur from cysteine, Nfs1 forms a covalently bound persulfide intermediate, which can be radiolabeled by addition of \textsuperscript{35}S-cysteine to mitochondrial extracts (Wiedemann et al., 2006). After incubation with \textsuperscript{35}S-cysteine, we analyzed the mitochondrial protein complexes by blue native electrophoresis. A major \textsuperscript{35}S-labeled protein complex of 230 kDa was detected (Figure 5C, top panel, lane 1). This protein complex represents the ISD machinery, since it was not detected in isolated mitochondrial extract from an isd11-1 mutant strain (Figure 5C, top panel, lane 2) (Wiedemann et al., 2006). Following this procedure, we could detect \textsuperscript{35}S-labeled Nfs1 on a nonreducing SDS-PAGE. We confirmed that the radiolabeled band resembles Nfs1 by two observations. First, this band was not labeled in isolated mitochondrial extract from an isd11-1 mutant strain (Figure 5C, bottom panel, lane 4). Second, it was shifted in size in a mitochondrial extract from cells expressing His-tagged Nfs1 (Figure 5C, bottom panel, lane 6). We combined this \textsuperscript{35}S-cysteine-labeling approach with the affinity purification via Cox4\textsubscript{His}. The radiolabeled Nfs1 protein and the radiolabeled ISD complex were coeluted with Cox4\textsubscript{His} (Figure 5D, lane 4). We conclude that the respiratory chain associates with a fraction of active and assembled ISD complex.

**Nfs1 and Isd11 bind selectively to respiratory chain supercomplexes**

The cytochrome bc\textsubscript{1} complex and the cytochrome c oxidase form respiratory chain supercomplexes in yeast mitochondria (Cruciat et al., 2000; Schägger and Pfeiffer, 2000; Zara et al., 2007; Böttinger et al., 2012). We wondered whether Nfs1 and Isd11 interact with either complex III or complex IV. A well-established approach to discriminate between binding of a protein to cytochrome bc\textsubscript{1} complex or cytochrome c oxidase is the lysis of mitochondrial membranes with dodecylmaltoside. In contrast to lysis with digitonin, the association of complex III and complex IV is disrupted in the presence of dodecylmaltoside (Schägger and Pfeiffer, 2000; Chen et al., 2012; Vukotic et al., 2012). Consequently, we lost the copurification of subunits (Cyt1, Qcr8, Rip1) and complex IV (Cox1, Cox2, Cox4) with Cor1\textsubscript{TAP} (Figure 6A, lane 4. Unexpectedly, Nfs1 was not detected in the elution fractions of both Cox4\textsubscript{His} and Cor1\textsubscript{TAP} (Figure 6B, lanes 4 and 8). To exclude the possibility that lysis with dodecylmaltoside unspecifically affects the binding of the ISD complex to the respiratory chain complexes, we analyzed this interaction in yeast mutant mitochondria deficient of either complex III (cor1\Delta) or complex IV (cox4\Delta) (Frazier et al., 2006; Zara et al., 2007; Böttinger et al., 2012).  

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**FIGURE 2:** Nfs1 and Isd11 bind to complex III of the respiratory chain. (A) Wild-type and Cor1\textsubscript{TAP} mitochondria were lysed with digitonin and incubated with IgG Sepharose. Load (3%) and elution fractions (100%) were analyzed by SDS–PAGE followed by immunodetection with the indicated antisera. (B) Wild-type and Sdh\textsubscript{4ProA} mitochondria were lysed with digitonin and incubated with IgG Sepharose. Load (3%) and elution fractions (100%) were analyzed by SDS–PAGE followed by immunodetection with the indicated antisera.

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grown on permissive conditions, and mitochondria were isolated (Wiedemann et al., 2006). The steady-state levels of Isd11 were strongly reduced in the mutant mitochondria, whereas the content of Nfs1 was mildly decreased compared with wild-type mitochondria (Figure 4A) as reported (Wiedemann et al., 2006). Subunits of complex III (Cyt1, Rip1) and complex IV (Cox1, Cox4, Cox9) are present in similar amounts in isd11-1 and wild-type mitochondria (Figure 4A). We studied the integrity of respiratory chain complexes by blue native electrophoresis. The cytochrome bc\textsubscript{1} complex forms a dimer that can associate with one or two copies of the cytochrome c oxidase (Cruciat et al., 2000; Schägger and Pfeiffer, 2000; Zara et al., 2007; Böttinger et al., 2012). The formation of the respiratory chain supercomplexes was not affected in isd11-1 mutant mitochondria (Figure 4B). We used communoprecipitation with Nfs1-specific antibodies to study the association of Nfs1 with respiratory chain components. Strikingly, the copurification of complex III (Qcr8, Rip1) and complex IV (Cox1, Cox2) subunits was blocked in isd11-1 mitochondria, although Nfs1 was coprecipitated to comparable amounts (Figure 4C, lanes 5 and 6). We conclude that Isd11 stabilizes the binding of Nfs1 to respiratory chain complexes.
et al., 2013). In cor1Δ mitochondria only assembled complex IV was detected on blue native PAGE, but no complex III was present (Figure 6B, lanes 1 and 4). Similarly, in cox4Δ mitochondria complex IV was absent, while complex III dimer was formed (Figure 6B, lanes 3 and 6) (Frazier et al., 2006; Zara et al., 2007; Böttinger et al., 2013). Thus, we lysed these mutant mitochondria with digitonin to analyze binding of Nfs1 to the individual complex III and complex IV via coimmunoprecipitation with Nfs1-specific antibodies (Figure 6C). Strikingly, the coimmunoprecipitation of Cox1 (complex IV), Rip1, and Qcr8 (complex III) with Nfs1-specific antibodies was abolished in cor1Δ and cox4Δ mitochondria, respectively (Figure 6C, lanes 7 and 8). We conclude that Nfs1 of the ISD complex does not efficiently bind to the individual complexes III and IV of the respiratory chain. This opens the exciting possibility that the ISD complex interacts selectively with respiratory chain supercomplexes. To address this point, we analyzed the pull down via Nfs1His by blue native electrophoresis. Only supercomplexes consisting of the cytochrome bc1 complex and cytochrome c oxidase but not the complex III dimer were copurified with His-tagged Nfs1 (Figure 6D, lanes 6 and 12). In particular, we found respiratory chain supercomplexes consisting of complex III dimer and two copies of complex IV in the elution fraction, while the supercomplex composed of a complex III dimer associated with one complex IV was coeluted to a smaller amount (Figure 6, lanes 6 and 12). Altogether, we discovered that the cysteine desulfurase Nfs1 and Isd11 bind specifically to respiratory chain supercomplexes in yeast mitochondria.

**DISCUSSION**

We identified a novel interaction of the respiratory chain with the iron–sulfur cluster assembly machinery. A fraction of the ISD complex binds selectively to respiratory chain supercomplexes of cytochrome bc1 complex and cytochrome c oxidase but not to the individual complexes. The respiratory chain-bound ISD complex contains the cysteine desulfurase Nfs1, Isd11, and Acp1 and is active. Destabilization of the ISD complex compromises the association of Nfs1 with the respiratory chain. The binding of the active and assembled ISD complex to respiratory chain supercomplexes represents a novel link between mitochondrial energy metabolism and ISC formation.

Isd11 is a member of LYR family of proteins that fulfill various different functions in mitochondria. Some LYR proteins are components of the respiratory chain or function in the biogenesis of...
respiratory chain complexes (Angerer, 2015). Acp1 interacts with the signature Lyr motif of different partner proteins (Angerer et al., 2017). It binds covalently to a 4-phosphopantheine with a conjugated acyl chain, which contributes to its binding to Isd11 within the ISD complex (Boniecki et al., 2017; Cory et al., 2017). Structural and functional studies revealed that Acp1 and Isd11 exert stabilizing and regulatory functions for the ISD complex (Adam et al., 2006; Wiedemann et al., 2006; van Vranken et al., 2014; Strogolova et al., 2017). Interestingly, in human mitochondria, Acp1 associates with complex I of the respiratory chain, linking ISC formation to energy metabolism (Angerer et al., 2014; Fiedorczuk et al., 2016; Zhu et al., 2016). In addition, large-scale protein interactome studies point to an association of human NFS1 with subunits of complex I (Floyd et al., 2016; Huttlin et al., 2017). However, an association of the entire ISD machinery to complex I with subunits of complex I (Floyd et al., 2016; Huttlin et al., 2017) protein interactome studies point to an association of human NFS1 and Isd11 contributing to the binding of the ISD complex to respiratory chain supercomplexes composed of complex III and IV in yeast mitochondria.

In yeast, several interaction partners of cytochrome bc1 and cytochrome c oxidase have been described. Prominent examples are proteins that are important for the biogenesis of these respiratory chain complexes such as assembly factors, including proteins that are important for formation and stability of respiratory chain supercomplexes (Mick et al., 2007; Chen et al., 2012; Strogolova et al., 2012; Vukotic et al., 2012; Singhal et al., 2017). In addition, subunits of the presequence translocase of the inner membrane (TIM23 complex) bind to respiratory chain complexes (van der Laan et al., 2006; Wiedemann et al., 2007). The TIM23 complex mediates protein import into the mitochondrial matrix and inner membrane, which is driven by the membrane potential across the inner membrane (Endo et al., 2011; Hewitt et al., 2011; Neupert, 2015; Wenz et al., 2015a; Wiedemann and Pfanner, 2017). According to the current view, the coupling of the respiratory chain with the presequence translocase facilitates the establishment of a local membrane potential to drive protein transport via the TIM23 complex (van der Laan et al., 2006). Interestingly, the ADP/ATP carrier associates with both the TIM23 complex and the respiratory chain supercomplexes (Dienhart and Stuart, 2008; Mehnert et al., 2014). It was reported that the ADP/ATP carrier is important for the function and biogenesis of the respiratory chain complexes (Dienhart and Stuart, 2008). Thus, the respiratory chain supercomplexes provide a docking platform for the association of a number of different proteins that are important for the formation and function of the respiratory chain.

The ISD complex is crucial to generate sulfide ions, which will be combined with ferrous iron into [2S-2Fe] clusters on the scaffold protein Isu1. The activity of the ISD complex is an essential step for ISC assembly (Rouault and Tong, 2005; Lill, 2009; Shefel et al., 2010; Stemmler et al., 2010; Ciesielski and Craig, 2017). Destabilized ISD complex leads to decreased activity of ISC-containing proteins (Adam et al., 2006; Wiedemann et al., 2006). Many protein machineries that contain an iron–sulfur cluster play important roles for respiratory metabolism, including complexes II and III of the respiratory chain (Rouault and Tong, 2005; Shefel et al., 2010). We speculate that the connection of the ISD complex with the respiratory chain complexes is a mode to locally coordinate ISC formation to the increased demands under respiratory growth conditions. Under these conditions, the amounts of respiratory chain complexes are strongly increased, whereas the content of Nfs1 remain largely similar.
strains, have been described (Frazier et al., 2006; Wiedemann et al., 2006; Gebert et al., 2011; Böttinger et al., 2013). For the generation of the Nfs1HisΔ, Yfh1HisΔ, Isu1His, and Acp1His strains, a His3MX6 cassette encoding for a deca-histidine tag was chromosomally inserted before the stop codon of the corresponding open reading frame by using gene-specific primers for homologous recombination (Böttinger et al., 2013). Yeast strains were grown on YPG (1% [wt/vol] yeast extract, 2% [wt/vol] bacto peptone, 3% [vol/vol] glycerol), YPGAL (1% [wt/vol] yeast extract, 2% [wt/vol] bacto peptone, 2% [wt/vol] galactose), or YPS medium (1% [wt/vol] yeast extract, 2% [wt/vol] bacto peptone, 2% [wt/vol] sucrose) at 24–30°C. Mitochondria were isolated by differential centrifugation (Wenz et al., 2015b). In brief, yeast cells were harvested at early logarithmic growth phase, and the cell wall was disrupted by incubation with Zymolyase (Seikagaku) in 1.2 M sorbitol and 20 mM K2HPO4, pH 7.2. After extensive washing, the cell membrane of the yeast spheroplasts was opened mechanically by homogenizing with a glass potter in 0.6 M sorbitol, 10 mM Tris/HisCl, pH 7.4, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2% (wt/vol) bovine serum albumin (BSA). We removed cell debris by centrifugation at 2500 × g, and mitochondria were pelleted at 17,000 × g. Mitochondria were resuspended in SEM buffer (10 mM MOPS/KOH, pH 7.2, 1 mM EDTA, 250 mM sucrose) and stored in aliquots at −80°C until use.

Affinity purification

For affinity purification of His-tagged proteins mitochondrial membranes were lysed with lysis buffer (20 mM Tris/HCl, pH 7.4, 50 mM NaCl, 10% [vol/vol] glycerol, 0.1 mM EDTA) containing 1% (wt/vol) digitonin and 10 mM imidazole. Insoluble material was removed by centrifugation at 17,000 × g. The supernatant was incubated with Ni-NTA agarose (Qiagen) for 1 h at 4°C under constant rotation to allow binding of the His-tagged protein. After extensive washing with lysis buffer containing 0.1% (wt/vol) digitonin and 40 mM imidazole, bound proteins were eluted with lysis buffer containing 250–500 mM imidazole and 0.1% (wt/vol) digitonin. For purification of associated proteins via Cor1TAP and Sdh4ProtA, mitochondrial membranes were solubilized with lysis buffer containing 1% (wt/vol) digitonin. The soluble fraction was incubated with immunoglobulin G (IgG) Sepharose (GE-Healthcare) for 1 h under constant rotation at 4°C. Subsequently, the beads were washed and incubated over night at 4°C with TEV protease to release Sdh4 and Cor1 from the fused Protein A-tag. Affinity purifications in the presence of dodecylmaltoside instead of digitonin were performed essentially as

(Morgenstern et al., 2017). Interestingly, mitochondrial Nfs1 binds to respiratory chain complexes under respiratory compared with fermentative growth conditions. Thus, the molecular coupling of ISC formation to mitochondrial respiration could represent a novel mode to adjust ISC formation to physiological conditions.

MATERIALS AND METHODS

Yeast strains, growth conditions, and isolation of mitochondria

The yeast strains Cox4His, Cox4His mss51Δ, Cor1TAP, Sdh4ProtA, isd11-1, cor1Δ, and cox4Δ, as well as their corresponding wild-type
Isd11 and Acp1 precursors were incubated with isolated mitochondria in import buffer (3% [wt/vol] BSA, 250 mM sucrose, 5 mM methionine, 80 mM KCl, 5 mM MgCl₂, 10 mM MOPS/KOH, pH 7.2, 2 mM KH₂PO₄) in the presence of 2 mM ATP and 2 mM NADH for different time points. The import was stopped by addition of an AVO mixture (8 µM antimycin A, 1 µM valinomycin, 20 µM oligomycin final concentrations) to deplete the membrane potential. Subsequent affinity purification of the imported ³⁵S-labeled precursor proteins via Cox4His-His was performed as described above.

**Cysteine labeling**

The cysteine-labeling experiments followed the described procedure (Wiedemann et al., 2006). In brief, isolated mitochondria were treated with chloramphenicol in 20 mM Tris/HCl, pH 7.4, 0.6 M sorbitol, 150 mM KCl, 12.5 mM MgCl₂ and 0.3% [wt/vol] BSA to block the mitochondrial protein biosynthesis. Subsequently, mitochondria were incubated with [³⁵S]cysteine in lysis buffer containing 0.4% [wt/vol] digitonin in the presence of 2 mM ATP and 2 mM NADH. The samples were analyzed by either nonreducing SDS–PAGE or blue native electrophoresis, and the radioactive signals were detected by autoradiography.

**Protein import into isolated mitochondria**

Mitochondria from a Cox4His strain were isolated as described above. We used a cell-free translation system based on rabbit reticulocyte lysate for coupled in vitro transcription and translation (Promega) of the radiolabeled Isd11 and Acp1 precursors. The protein synthesis was performed in the presence of [³⁵S]methionine to radiolabel the precursor proteins. The ³⁵S-labeled Lsd11 and Acp1 precursors were incubated with isolated mitochondria in import buffer (3% [wt/vol] BSA, 250 mM sucrose, 5 mM methionine, 80 mM KCl, 5 mM MgCl₂, 10 mM MOPS/KOH, pH 7.2, 2 mM KH₂PO₄) in the presence of 2 mM ATP and 2 mM NADH for different time points. The import was stopped by addition of an AVO mixture (8 µM antimycin A, 1 µM valinomycin, 20 µM oligomycin final concentrations) to deplete the membrane potential. Subsequent affinity purification of the imported ³⁵S-labeled precursor proteins via Cox4His-His was performed as described above.

**Coimmunoprecipitation**

For coimmunoprecipitation, antibodies from an Nfs1-specific antisera and the corresponding preimmune serum were covalently coupled to Protein A Sepharose (GE Healthcare) with dimethyl pimelimidate. Mitochondria were solubilized in lysis buffer containing 1% [wt/vol] digitonin and incubated with the antibody-covered Protein A Sepharose for 1.5 h under constant rotation at 4°C. After excessive washing of the beads with lysis buffer containing 0.2% [wt/vol] digitonin, bound proteins were eluted with 0.1 M glycine, pH 2.5. The elution samples were immediately neutralized by addition of Tris base.

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**FIGURE 6:** Nfs1 binds selectively to respiratory chain supercomplexes. (A) Left panel, wild-type and Cox4His-His mitochondria were lysed with dodecylmaltoside and incubated with Ni-NTA agarose. Load (3%) and elution fractions (100%) were analyzed by SDS–PAGE followed by immunodetection with the indicated antisera. Right panel, wild-type and Cor1ΔΔ mitochondria were lysed with dodecylmaltoside and incubated with IgG Sepharose. Load (3%) and elution fractions (100%) were analyzed by SDS–PAGE followed by immunodetection with the indicated antisera. (B) Wild-type, cor1Δ, and cor4Δ mitochondria were lysed with digitonin, and protein complexes were analyzed by blue native electrophoresis followed by immunodetection with the indicated antisera. III, cytochrome bc₁ complex; IV, cytochrome c oxidase. (C) Wild-type, cor1Δ, and cor4Δ mitochondria were lysed with digitonin and subjected to coimmunoprecipitation (Co-IP) with Nfs1-specific antibodies or the corresponding pre-immune serum. Load (1%) and elution fractions (100%) were analyzed by SDS–PAGE followed by immunodetection with the indicated antisera. (D) Wild-type and Nfs1His-His mitochondria were lysed with digitonin and incubated with Ni-NTA agarose. Load (1%) and elution fractions (100%) were analyzed by blue native electrophoresis followed by immunodetection with the indicated antisera.
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