Respiratory chain supercomplexes associate with the cysteine desulfurase 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 F_1F_O-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 desulfurase Nfs1 provides sulfur for ISC assembly and forms with partner proteins the ISC-biogenesis desulfurase 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.**

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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 F_1F_O-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;

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Abbreviations used: Acp1, acyl carrier protein 1; complex II, succinate dehydrogenase; complex III, cytochrome *bc1* complex; complex IV, cytochrome *c* oxidase; ISC, iron–sulfur cluster; ISD, ISC-biogenesis desulfurase; TIM23, presequence translocase of the inner mitochondrial membrane; TOM, translocase of the outer mitochondrial membrane.

Wenz *et al.*, 2015a; Neupert, 2015; Wiedemann and Pfanner, 2017). The assembly of mitochondria- and nucleus-encoded subunits into the respiratory chain complexes is a complicated process and involves a large set of assembly factors (Fox, 2012; Kim *et al.*, 2012; Soto *et al.*, 2012a; Herrmann *et al.*, 2013; Richter-Dennerlein *et al.*, 2015). The biogenesis of the respiratory chain complexes depends not only on the association of protein subunits but also on the insertion of several redox-active groups, including ISC, heme, and copper ions (Kim *et al.*, 2012; Soto *et al.*, 2012a). These reactive centers are crucial for electron transport within the respiratory chain.

The assembly of ISC involves several proteins in the mitochondrial matrix (Rouault and Tong, 2005; Lill, 2009; Sheftel *et al.*, 2010; Stemmler *et al.*, 2010; Ciesielski and Craig, 2017). The [2Fe2S] cluster is formed on the scaffold protein Isu1/Isu2 (Schilke *et al.*, 1999; Mühlenhoff *et al.*, 2003). Critical for the ISC assembly is the activity of Nfs1 that releases sulfur from cysteine (Kispal *et al.*, 1999; Mühlenhoff *et al.*, 2003). The stability of this cysteine desulfurase depends on its interaction with Isd11 and the acyl carrier protein Acp1 within the ISC-biogenesis desulfurase complex (ISD complex) (Adam *et al.*, 2006; Wiedemann *et al.*, 2006; Van Vranken *et al.*, 2016; Cory *et al.*, 2017; Boniecki *et al.*, 2017). Frataxin (Yfh1) was proposed to provide iron and to regulate the cysteine desulfurase activity (Webert *et al.*, 2014; Parent *et al.*, 2015). In addition, ferredoxin (Yah1) and the ferredoxin reductase (Arh1) form an electron transport chain that is required for ISC assembly (Lange *et al.*, 2000; Li *et al.*, 2001; Webert *et al.*, 2014). Subsequently, a dedicated Hsp70 chaperone system releases the newly synthesized ISC from Isu1 and specific factors transfer it onto apoproteins (Rouault and Tong, 2005; Lill, 2009; Kampinga and Craig, 2010; Sheftel *et al.*, 2010; Stemmler *et al.*, 2010; Ciesielski and Craig, 2017). The formation of ISC is crucial for respiratory growth. ISC form reactive centers in complex II and complex III of the respiratory chain and are required as a prosthetic group for the function of the citric acid cycle enzyme aconitase. Furthermore, the activity of the lipoic acid synthase depends on its bound ISC. Lipoic acid in turn forms a reactive center of the pyruvate dehydrogenase and of the oxoglutarate dehydrogenase of the citric acid cycle (Rouault and Tong, 2005; Sheftel *et al.*, 2010). In addition, the mitochondrial ISC assembly machinery is required for the formation of cytosolic ISC. The group of ISC-containing proteins, which depend on the cytosolic ISC assembly machinery, includes enzymes that are crucial for nuclear gene expression and maintenance (Rouault and Tong, 2005; Lill, 2009; Sheftel *et al.*, 2010; Stemmler *et al.*, 2010; Ciesielski and Craig, 2017). Consequently, mitochondrial ISC biogenesis is essential for cell viability under all growth conditions.

Here we report an unexpected association of the cysteine desulfurase Nfs1 and its partner proteins Isd11 and Acp1 of the ISD complex with complex III and complex IV of the respiratory chain. We demonstrate that the assembled and active ISD complex binds to the respiratory chain. Individual deletion of complex III and complex IV abolishes this association. Thus, we conclude that binding of the cysteine desulfurase machinery to the respiratory chain subunits depends on the presence of respiratory chain supercomplexes. We propose that this interaction resembles a novel mechanism to spatially coordinate iron–sulfur cluster formation with respiratory activity.

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 His tagging 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 cysteine desulfurase Nfs1 and its binding partner Isd11 of the ISC assembly machinery were efficiently copurified along with $Cox4_{His}$ (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 Pam18 in the $Cox4_{His}$ 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 $Cox4_{His}$. Interestingly, Nfs1 also binds to $Cox4_{His}$ 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 $Cox4_{His}$ 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 mtHsp70 (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 mtHsp70-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, Cyt1) do not interact with Cox4 in the *mss51* deletion mitochondria (Figure 1C, lane 6). In contrast, binding of mtHsp70 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 $Cor1_{TAP}$ (Figure 2A, lanes 4 and 8). Quantification reveals that a similar fraction of Nfs1 was bound to $Cor1_{TAP}$ and $Cox4_{His}$ (Figure 1B). As control, Tim23 was copurified along with $Cor1_{TAP}$ 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, Cyt1) 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.

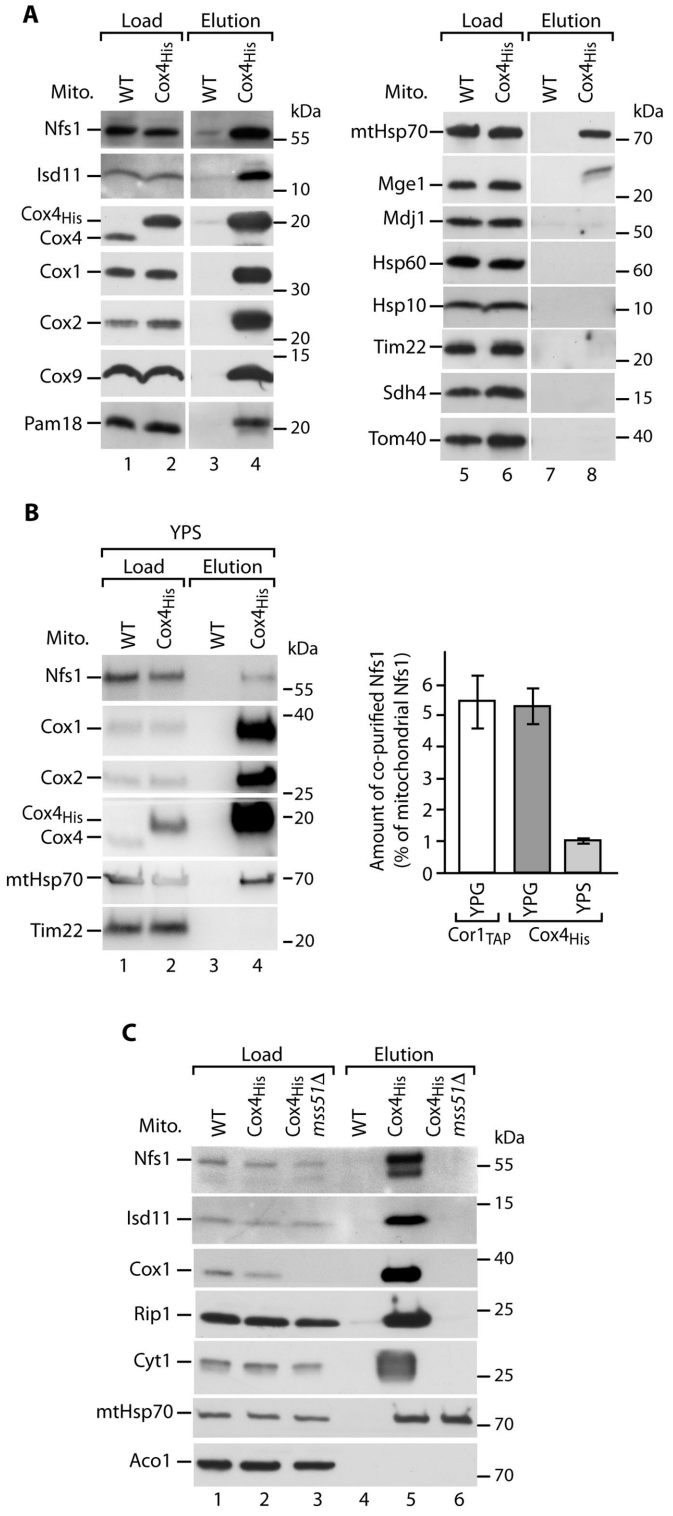

FIGURE 1: Respiratory chain complex IV associates with Nfs1 and Isd11. (A) Wild-type and $Cox4_{His}$ cells were grown under nonfermentative conditions with glycerol (YPG) as a carbon source. Isolated mitochondria were lysed with digitonin and incubated with Ni-NTA agarose. Load (3%) and elution fractions (100%) were analyzed by SDS–PAGE followed by immunodetection with the indicated antisera. (B) Left panel, wild-type and $Cox4_{His}$ 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 $Cor1_{TAP}$ and $Cox4_{His}$ from cells grown under respiratory (YPG) and fermentative growth conditions (YPS). Depicted are mean values of four ($Cor1_{TAP}$, YPG), five ($Cox4_{His}$, YPG), and four ($Cox4_{His}$, YPS) independent experiments as shown in A and B and Figure 2A with the corresponding SEM. (C) Wild-type, $Cox4_{His}$, and $Cox4_{His}$ *mss51*Δ 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.

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 $Nfs1_{His}$ (Figure 3A, lanes 4 and 8). To rule out the possibility that tagging of proteins lead to artificial protein–protein interaction, we used a coimmunoprecipiation 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 $Acp1_{His}$, revealing that the integrity of the ISD complex is intact. Similarly to our affinity purifications via $Nfs1_{His}$, several subunits of complex III (Qcr8, Rip1) and complex IV (Cox1, Cox2) of the respiratory chain were co-eluted with $Acp1_{His}$, whereas subunits of complex II (Sdh1, Sdh4) and several matrix proteins were not present in the $Acp1_{His}$-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

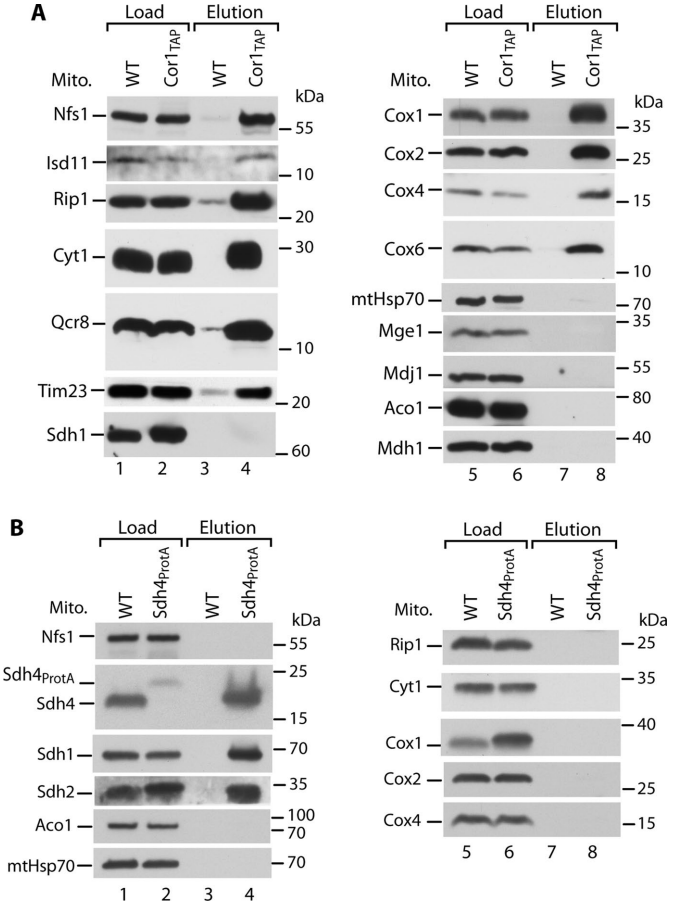

FIGURE 2: Nfs1 and Isd11 bind to complex III of the respiratory chain. (A) Wild-type and $Cor1_{TAP}$ mitochondria were lysed with digitonin and incubated with IgG Sepharose. Load (3%) and elution fractions (100%) were analyzed by SDS–PAGE followed by immunodetection with the indicated antisera. (B) Wild-type and $Sdh4_{ProtA}$ 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.

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 *bc1* 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 coimmunoprecipiation 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.

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 $Cox4_{His}$ 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 $Cox4_{His}$-bound complex. We synthesized ^{35}S-labeled Isd11 and Acp1 in a cell-free translation system and individually imported the proteins into isolated $Cox4_{His}$ 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 $Cox4_{His}$ (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 [^{35}S]cysteine to mitochondrial extracts (Wiedemann *et al.*, 2006). After incubation with [^{35}S]cysteine, we analyzed the mitochondrial protein complexes by blue native electrophoresis. A major ^{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 ^{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 *isd11-1* (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 [^{35}S]cysteine-labeling approach with the affinity purification via $Cox4_{His}$. The radiolabeled Nfs1 protein and the radiolabeled ISD complex were coeluted with $Cox4_{His}$ (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 *bc1* 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 *bc1* 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) of the cytochrome *bc1* complex with $Cox4_{His}$ (Figure 6A, lane 4) and subunits of complex IV (Cox1, Cox2, Cox4) with $Cor1_{TAP}$ (Figure 6A, lane 8). Unexpectedly, Nfs1 was not detected in the elution fractions of both $Cox4_{His}$ and $Cor1_{Tap}$ (Figure 6A, 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*Δ) or complex IV (*cox4*Δ) (Frazier *et al.*, 2006; Zara *et al.*, 2007; Böttinger

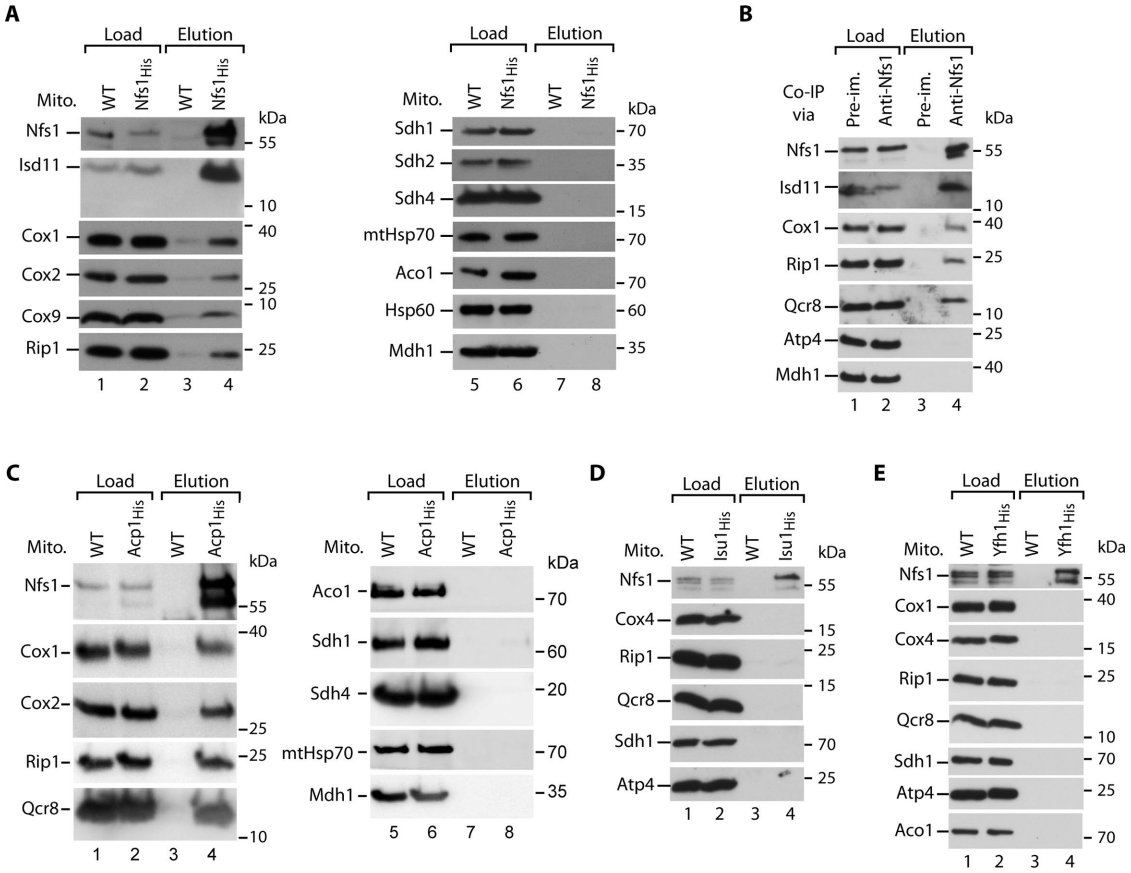

FIGURE 3: Respiratory chain complexes interact with specific ISD components. (A) Wild-type and $Nfs1_{His}$ mitochondria were lysed with digitonin and incubated with Ni-NTA agarose. Load (1%) and elution fractions (100%) were analyzed by SDS–PAGE followed by immunodetection with the indicated antisera. (B) Wild-type mitochondria were lysed and subjected to coimmunoprecipitation (Co-IP) with Nfs1-specific antibodies or antibodies of the corresponding preimmune serum. Load (1%) and elution fractions (100%) were analyzed by SDS–PAGE followed by immunodetection with the indicated antisera. For the detection of Nfs1 and Isd11 6% of the load fraction was analyzed. (C–E) Wild-type and $Acp1_{His}$ (C), $Isu1_{His}$ (D), or $Yfh1_{His}$ (E) 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.

et al., 2013). In *cor1*Δ 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 *cox4*Δ 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 *cor1*Δ and *cox4*Δ 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 $Nfs1_{His}$ 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

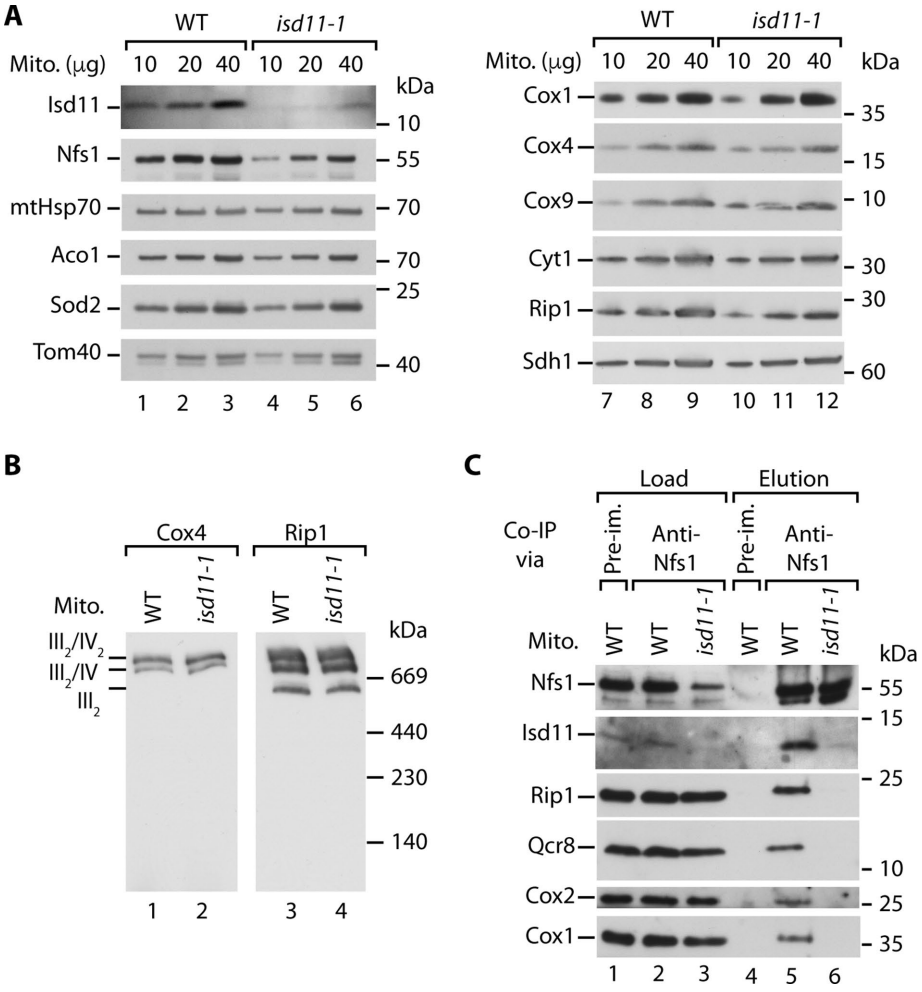

FIGURE 4: Isd11 is crucial for Nfs1 to bind to the respiratory complexes. (A) The indicated amounts of mitochondrial proteins from wild-type and *isd11-1* strains were analyzed by SDS–PAGE and immunodetection with the indicated antisera. (B) Respiratory chain complexes from wild-type and *isd11-1* mitochondria were analyzed by blue native electrophoresis and immunodetection with the indicated antisera. III, cytochrome bc_1 complex; IV, cytochrome *c* oxidase. (C) Wild-type and *isd11-1* mitochondria were lysed with digitonin and subjected to coimmunoprecipitation (Co-IP) with Nfs1-specific antibodies or antibodies from the corresponding preimmune serum. Load (1%) and elution fractions (100%) were analyzed by SDS–PAGE followed by immunodetection with the indicated antisera. For the detection of Nfs1 and Isd11 6% of the load fraction was analyzed.

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.*, 2016; Boniecki *et al.*, 2017; Cory *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 in human mitochondria was not shown so far. Mitochondria from *S. cerevisiae* lack complex I. Since the integrity of the ISD complex is crucial for the binding of Nfs1 to the respiratory chain, one can speculate that the regulatory ISD components Acp1 and Isd11 contributes 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; Sheftel *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; Sheftel *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

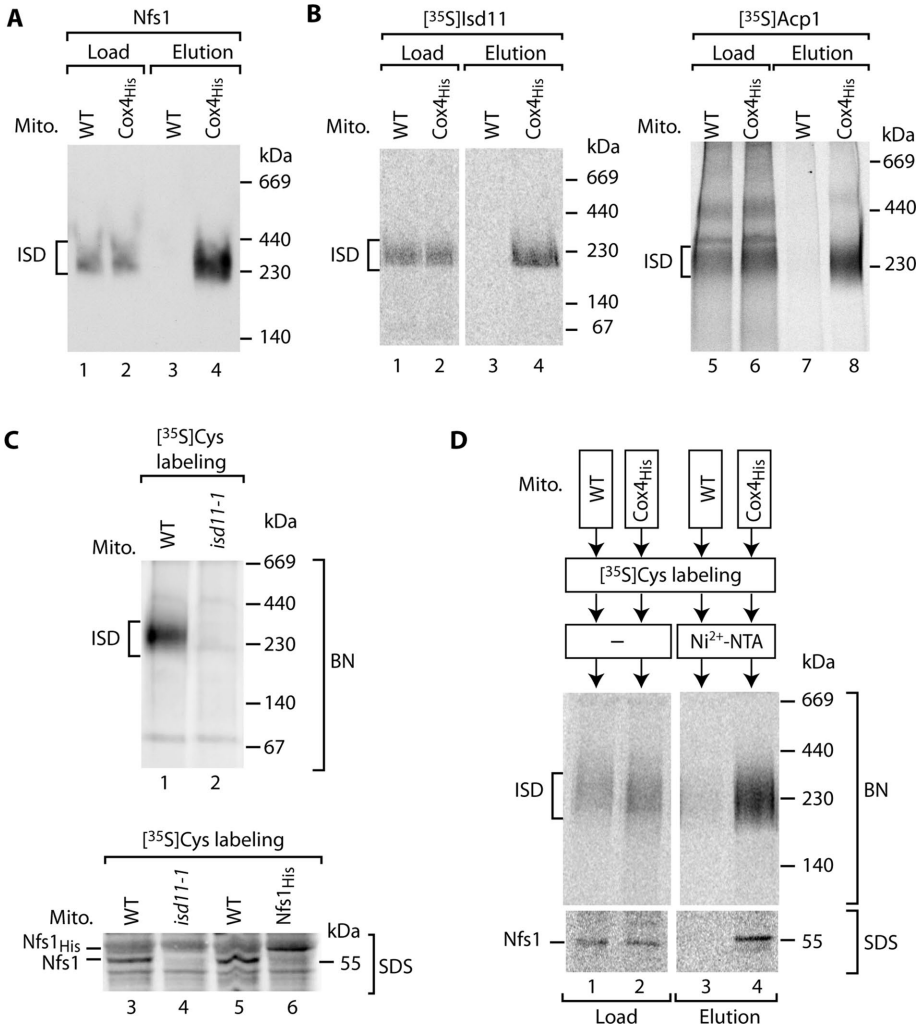

FIGURE 5: The active cysteine desulfurase complex binds to respiratory chain complexes. (A) Wild-type and $Cox4_{His}$ mitochondria were lysed with digitonin and subjected to affinity purification via Ni-NTA agarose. Load (1%) and elution (100%) fractions were analyzed by blue native electrophoresis followed by immunodetection with the indicated antiserum. ISD, ISC-biogenesis desulfurase complex. (B) ^{35}S-labeled Isd11 (lanes 1–4) and Acp1 (lanes 5–8) were imported into isolated wild-type or $Cox4_{His}$ mitochondria. Subsequently, the mitochondria were lysed with digitonin and subjected to affinity purification via Ni-NTA agarose. Load (3% for Isd11; 6% for Acp1) and elution (100%) fractions were analyzed by blue native electrophoresis followed by autoradiography. (C) Mitochondrial lysates from wild-type, *isd11-1*, and $Nfs1_{His}$ strains were incubated with [^{35}S]cysteine. The samples were analyzed by blue native electrophoresis (top panel) or by nonreducing SDS–PAGE (bottom panel). ^{35}S-labeled proteins were detected by autoradiography. (D) Mitochondrial lysates from wild-type and $Cox4_{His}$ strains were incubated with [^{35}S]cysteine and subjected to affinity purification via Ni-NTA agarose. Load (1%) and elution (100%) fractions were analyzed by blue native electrophoresis (top panel) or nonreducing SDS–PAGE (bottom panel) followed by autoradiography.

(Morgenstern *et al.*, 2017). Interestingly, more 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 $Cox4_{His}$, $Cox4_{His}$ *mss51*Δ, $Cor1_{TAP}$, $Sdh4_{ProtA}$, *isd11-1*, *cor1*Δ, and *cox4*Δ, as well as their corresponding wild-type strains, have been described (Frazier *et al.*, 2006; Wiedemann *et al.*, 2006, 2007; Gebert *et al.*, 2011; Böttinger *et al.*, 2013). For the generation of the $Nfs1_{His}$, $Yfh1_{His}$, $Isu1_{His}$, and $Acp1_{His}$ 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 K_2HPO_4, pH 7.2. After excessive 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/HCl, 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 $Cor1_{TAP}$ and $Sdh4_{ProtA}$, 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

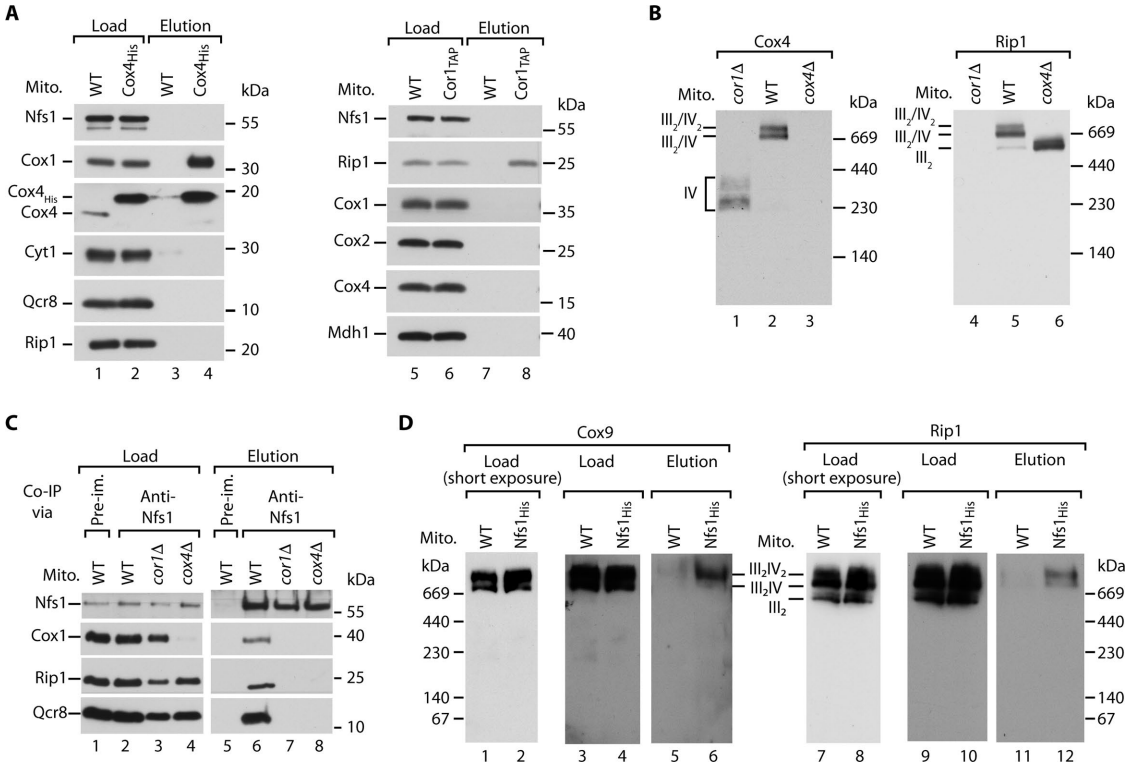

FIGURE 6: Nfs1 binds selectively to respiratory chain supercomplexes. (A) Left panel, wild-type and $Cox4_{His}$ mitochondria were lysed with dodecylmaltoside and incubated with Ni-NTA agarose. Load (3%) and elution fractions (100%) were analyzed by SDS–PAGE followed by immunodetection with the indicated antisera. Right panel, wild-type and $Cor1_{TAP}$ 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, *cor1*Δ, and *cox4*Δ mitochondria were lysed with digitonin, and protein complexes were analyzed by blue native electrophoresis followed by immunodetection with the indicated antisera. III, cytochrome bc_1 complex; IV, cytochrome *c* oxidase. (C) Wild-type, *cor1*Δ, and *cox4*Δ 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 $Nfs1_{His}$ 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.

described above. Here, 0.6% (wt/vol) dodecylmaltoside was used to lyse the mitochondrial membranes.

Coimmunoprecipitation

For coimmunoprecipitation, antibodies from an Nfs1-specific antiserum 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.

Protein import into isolated mitochondria

Mitochondria from a $Cox4_{His}$ 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 [^{35}S]methionine to radiolabel the precursor proteins. The ^{35}S-labeled 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 $MgCl_2$, 10 mM MOPS/KOH, pH 7.2, 2 mM KH_2PO_4) 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 ^{35}S-labeled precursor proteins via $Cox4_{His}$ 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 $MgCl_2$ and 0.3% (wt/vol) BSA to block the mitochondrial protein biosynthesis. Subsequently, mitochondria were incubated with [^{35}S]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.

Miscellaneous

All antibodies used for immunodetection have been described (Wiedemann *et al.*, 2006; Gebert *et al.*, 2011; Böttinger *et al.*, 2013). The immunosignals were extensively controlled by their absence in mitochondria from the corresponding deletion strains or by their upshift in strains expressing tagged versions of the protein. For immunodetection proteins were transferred on polyvinylidene difluoride membranes by semi-dry Western blotting. The immunosignals were detected by enhanced chemoluminescence (Haan and Behrmann, 2007). We used the Multi Gauge software Version 3.2 (Fujifilm) to quantify Western blot signals. Blue native electrophoresis was performed as described (Schägger and Pfeiffer, 2000; Böttinger *et al.*, 2013). For size estimation, a protein standard was used that include thyreoglobin (669 kDa), ferritin (440 kDa), catalase (230 kDa), lactate dehydrogenase (140 kDa), and BSA (67 kDa). For SDS–PAGE, we used two commercial protein molecular weight standards: Novex Sharp Prestained (Invitrogen) and PageRuler Prestained Protein Ladder (Thermofisher). Radioactive signals were detected after exposure to phosphor screens using phosphor imagers (Storm 840, GE Healthcare; FLA 9000, FujiFilm).

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