Molecular Cell
Short Article

Dual Function of Sdh3 in the Respiratory Chain and TIM22 Protein Translocase of the Mitochondrial Inner Membrane

Natalia Gebert,[1] Michael Gebert,[1,2] Silke Oeljeklaus,[3] Karina von der Malsburg,[1] David A. Stroud,[1,2] Bogusz Kulawiak,[1] Christophe Wirth,[1] René P. Zahedi,[5] Pavel Dolezal,[6] Sebastian Wiese,[3,4] Oliver Simon,[5] Agnes Schulze-Specking,[1] Kaye N. Truscott,[1,7] Albert Sickmann,[5] Peter Rehling,[8] Bernard Guiard,[9] Carola Hunte,[1,4] Bettina Warscheid,[3,4] Martin van der Laan,[1,4] Nikolaus Pfanner,[1,4,*] and Nils Wiedemann[1,4,*]
[1]Institut für Biochemie und Molekularbiologie, ZBMZ
[2]Fakultät für Biologie
[3]Institut für Biologie II, Fakultät für Biologie, Funktionelle Proteomik
[4]BIOSS Centre for Biological Signalling Studies
Universität Freiburg, 79104 Freiburg, Germany
[5]Leibniz-Institut für Analytische Wissenschaften ISAS e.V., 44139 Dortmund, Germany
[6]Department of Parasitology, Charles University, 12844 Prague, Czech Republic
[7]Department of Biochemistry, La Trobe University, Melbourne 3086, Australia
[8]Abteilung Biochemie II, Universität Göttingen, 37073 Göttingen, Germany
[9]Centre de Génétique Moléculaire, CNRS, 91190 Gif-sur-Yvette, France
*Correspondence: nikolaus.pfanner@biochemie.uni-freiburg.de (N.P.), nils.wiedemann@biochemie.uni-freiburg.de (N.W.)
DOI 10.1016/j.molcel.2011.09.025

SUMMARY

The mitochondrial inner membrane harbors the complexes of the respiratory chain and translocase complexes for precursor proteins. We have identified a further subunit of the carrier translocase (TIM22 complex) that surprisingly is identical to subunit 3 of respiratory complex II, succinate dehydrogenase (Sdh3). The membrane-integral protein Sdh3 plays specific functions in electron transfer in complex II. We show by genetic and biochemical approaches that Sdh3 also plays specific functions in the TIM22 complex. Sdh3 forms a subcomplex with Tim18 and is involved in biogenesis and assembly of the membrane-integral subunits of the TIM22 complex. We conclude that the assembly of Sdh3 with different partner proteins, Sdh4 and Tim18, recruits it to two different mitochondrial membrane complexes with functions in bioenergetics and protein biogenesis, respectively.

INTRODUCTION

Mitochondria play crucial functions in energy conversion and metabolism of eukaryotic cells. The mitochondrial inner membrane is a highly protein-rich membrane and harbors the complexes of the respiratory chain, the ATP synthase, and numerous metabolite carriers. Most mitochondrial proteins are synthesized as precursors in the cytosol and imported into mitochondria by the general translocase of the outer membrane (TOM) and two translocases of the inner membrane (TIM) (Dolezal et al., 2006; Neupert and Herrmann, 2007; Chacinska et al., 2009). The presequence translocase (TIM23 complex) mediates the import of preproteins with cleavable N-terminal presequences, whereas the carrier translocase (TIM22 complex) is responsible for the insertion of noncleavable inner membrane proteins.

The TIM22 complex consists of three membrane-integral subunits, Tim54, Tim22, and Tim18, and the peripheral chaperone complex Tim9-Tim10-Tim12 (Neupert and Herrmann, 2007; Gebert et al., 2008; Lionaki et al., 2008). Tim22 forms the channel for preprotein insertion (Rehling et al., 2003). Tim54 interacts with the peripheral TIM chaperone complex and promotes protein assembly (Hwang et al., 2007; Wagner et al., 2008). Tim18 is involved in the assembly of Tim54 into the TIM22 complex. Tim18 is homologous to subunit 4 of succinate dehydrogenase (SDH), also termed respiratory chain complex II (Kerscher et al., 2000; Koehler et al., 2000; Lemire and Oyedotun, 2002), though the functional relevance of the homology is unknown.

For this report, we attempted to analyze a possible role of Sdh4 in the TIM22 complex, but found that Sdh3, not Sdh4, plays a specific role in the translocase. Sdh3, an integral membrane protein with three transmembrane segments, does not show homology to any known mitochondrial translocase component. Genetic and biochemical analysis revealed that Sdh3 is a genuine component of the TIM22 complex and required for biogenesis and assembly of the membrane-integral subunits of the translocase. Thus, Sdh3 is located in both respiratory chain complex II and the carrier translocase.

RESULTS

Suppression of a *tim22* Yeast Mutant by *SDH3*, but Not *SDH4*

We performed a genetic analysis of the yeast mitochondrial TIM22 machinery and generated a temperature-sensitive mutant of *TIM22*. We asked if overexpression of other translocase

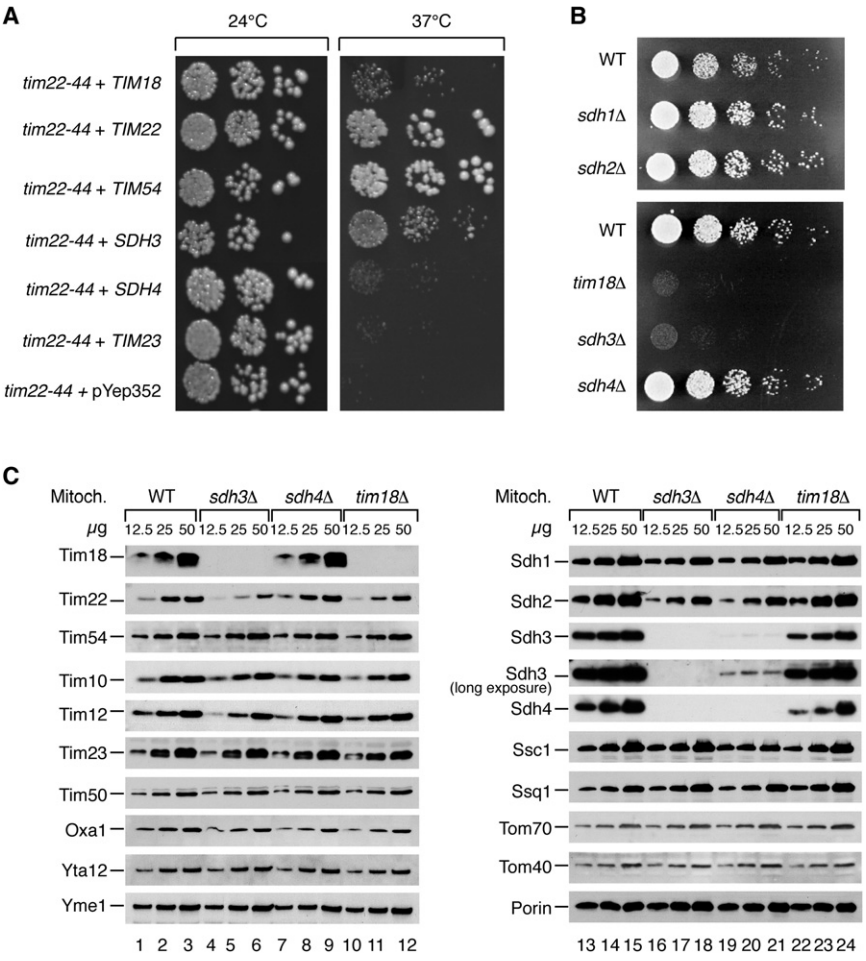

Figure 1. Overexpression of Sdh3 Suppresses a *tim22* Yeast Mutant

(A) The *tim22-44* yeast mutant strain was transformed with the indicated plasmids and grown on YPEG at 24°C or 37°C.

(B) The indicated yeast strains were grown at 30°C on YPD plates in the presence of ethidium bromide. WT, wild-type.

(C) Mitochondria (microgram protein) isolated from the indicated yeast strains grown at 37°C on YPEG were analyzed using SDS-PAGE and western blotting.

subunits was able to suppress the *tim22-44* mutant phenotype. We tested the membrane-integral subunits of the TIM22 complex (Tim18, Tim22, Tim54); expression of Tim22 or Tim54 from a high-copy number plasmid fully rescued growth of the mutant at 37°C (Figure 1A). As control, we also expressed Tim23 of the presequence translocase, as well as the two membrane-integral subunits of respiratory complex II, Sdh3 and Sdh4 (Oyedotun and Lemire, 2004; Sun et al., 2005). Whereas neither overexpression of Sdh4 nor Tim23 rescued growth of the mutant, overexpression of Sdh3 partially suppressed the growth defect of the *tim22-44* mutant cells at 37°C (Figure 1A).

To obtain further in vivo evidence that Sdh3 behaves in a way different from the other subunits of the SDH complex, we made use of the observation that most yeast mutants, including mutants lacking respiratory chain subunits, can grow on fermentable medium without mitochondrial DNA. A number of yeast mutants, termed petite negative mutants, cannot grow in the absence of mitochondrial DNA, including mutants that affect components of the carrier pathway (Kerscher et al., 2000; Dunn and Jensen, 2003; Dunn et al., 2006). We grew yeast deletion mutants on fermentable medium in the presence of ethidium bromide to induce the loss of mitochondrial DNA (Dunn et al., 2006). *sdh3Δ* cells indeed behaved like *tim18Δ* cells and stopped growth, whereas yeast mutants lacking one of the other SDH subunits tolerated the loss of mitochondrial DNA (Figure 1B).

In *sdh3Δ* mitochondria, Sdh4 as well as Tim18 were virtually absent, and the levels of Sdh2 and Tim22 were reduced (Figure 1C). For comparison, lack of Sdh4 led to a large decrease in Sdh3 levels (Oyedotun and Lemire, 1999), but did not affect the levels of TIM proteins. The level of Tim22 was moderately reduced in *tim18Δ* mitochondria (Figure 1C). We conclude that the lack of Sdh3 leads to a large decrease in the levels not only of Sdh4, but also of Tim18.

Sdh3 Is Required for Assembly of Tim18 into the TIM22 Complex

We used blue native electrophoresis to analyze the TIM22 complex and the SDH complex from *sdh3Δ* and *sdh4Δ* mitochondria upon lysis of the membranes with digitonin (to minimize indirect effects for the analysis shown in Figures 2 and 3, the cells were grown at lower temperature such that the level of Tim22 in *sdh3Δ* mitochondria was only moderately reduced; the level of Tim18 was still strongly reduced [Figure S1A]). As expected, the SDH complex migrating at ~200 kDa was lost upon lack of Sdh3 or Sdh4 (Oyedotun and Lemire, 1999), yet the TIM22 complex of ~300 kDa was only affected by the lack of Sdh3, not of Sdh4 (Figure 2A). In *sdh3Δ* mitochondria, the TIM22 complex was present in lower amounts, migrated at ~250 kDa, and was termed TIM22′ (Figure 2A). In *tim18Δ* mitochondria, the TIM22 complex was similarly affected (Figure 2B) (Kerscher et al., 2000; Koehler et al., 2000; Gebert et al., 2008; Wagner et al., 2008).

Our results suggest that Sdh3 may be required for the biogenesis of Tim18. We synthesized radiolabeled Tim18 precursor in reticulocyte lysate and imported it into isolated mitochondria. In wild-type mitochondria as well as in *tim18Δ* mitochondria, Tim18 assembled into the 300 kDa TIM22 complex (Figure 2C) (Wagner et al., 2008). In *sdh3Δ* mitochondria, [^{35}S]Tim18 assembled into neither the TIM22 complex nor the smaller TIM22′ complex. In mitochondria isolated from

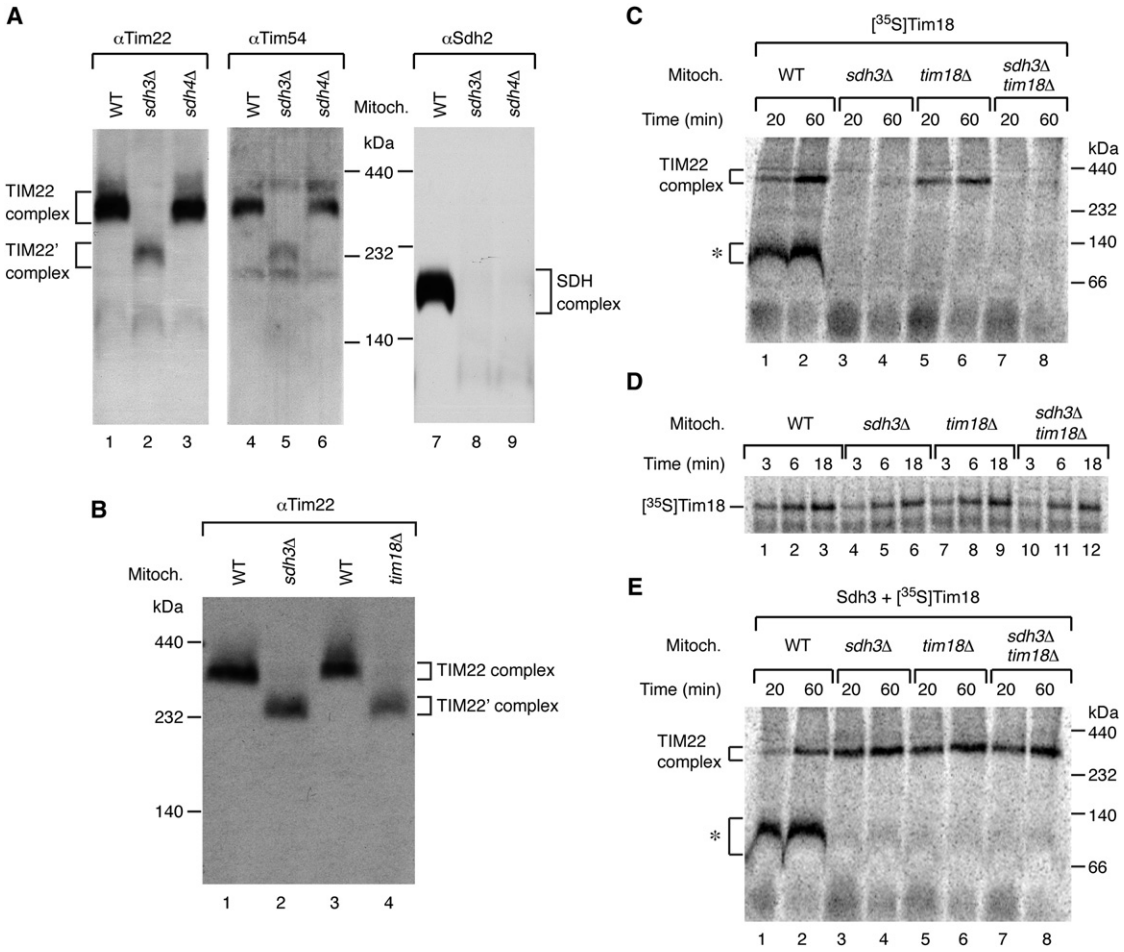

Figure 2. Sdh3 Is Required for the Assembly of Tim18

(A) Isolated mitochondria were lysed by digitonin and analyzed by blue native electrophoresis and western blotting.

(B) Isolated mitochondria were analyzed by blue native electrophoresis.

(C) [^{35}S]Tim18 was imported into isolated mitochondria. Mitochondria were solubilized in digitonin and analyzed by blue native electrophoresis and autoradiography. Asterisk, assembly intermediate of Tim18.

(D) [^{35}S]Tim18 was imported into isolated mitochondria, followed by treatment with proteinase K and separation by SDS-PAGE.

(E) [^{35}S]Tim18 was imported and analyzed as described in (C) with the exception that chemical amounts of Sdh3 were present during the import reaction. See also Figure S1.

a *sdh3Δ tim18Δ* double deletion mutant, the assembly of [^{35}S]Tim18 was similarly inhibited (Figure 2C).

We asked whether Sdh3 was required for translocation of the Tim18 precursor into mitochondria or the subsequent assembly into the TIM22 complex. The Tim18 precursor is imported via the presequence translocase, the presequence is removed, and the imported mature-sized Tim18 protein is protected against proteases added to mitochondria (Kerscher et al., 2000; Koehler et al., 2000). *sdh3Δ* mitochondria as well as *tim18Δ* mitochondria imported [^{35}S]Tim18 to a protease-protected location (Figure 2D); the majority of imported [^{35}S]Tim18 became resistant to alkaline extraction (Figure S1B), indicating membrane integration of the protein. The efficiency of translocation into *sdh3Δ* mitochondria was only moderately reduced compared to wild-type mitochondria and thus could not explain the strong defect of Tim18 assembly into the TIM22 complex.

To directly show that Sdh3 was required for the assembly of Tim18, we synthesized chemical amounts of nonradiolabeled Sdh3 precursor in a wheat germ-based translation system and imported it into *sdh3Δ* mitochondria together with radiolabeled Tim18 precursor. Under these conditions, [^{35}S]Tim18 efficiently assembled into the TIM22 complex (Figure 2E). In *sdh3Δ tim18Δ* mitochondria, the import of [^{35}S]Tim18 together with chemical amounts of Sdh3 also led to efficient assembly into the TIM22 complex. These results indicate that Sdh3 is required for the assembly of Tim18 into the TIM22 complex.

Mitochondria Lacking Sdh3 Are Impaired in the Import of Substrates of the Carrier Pathway

We asked if Sdh3 also plays a role in the biogenesis of the other membrane-integral subunits of the TIM22 complex. We synthesized the ^{35}S-labeled precursor of Tim54 and imported it into

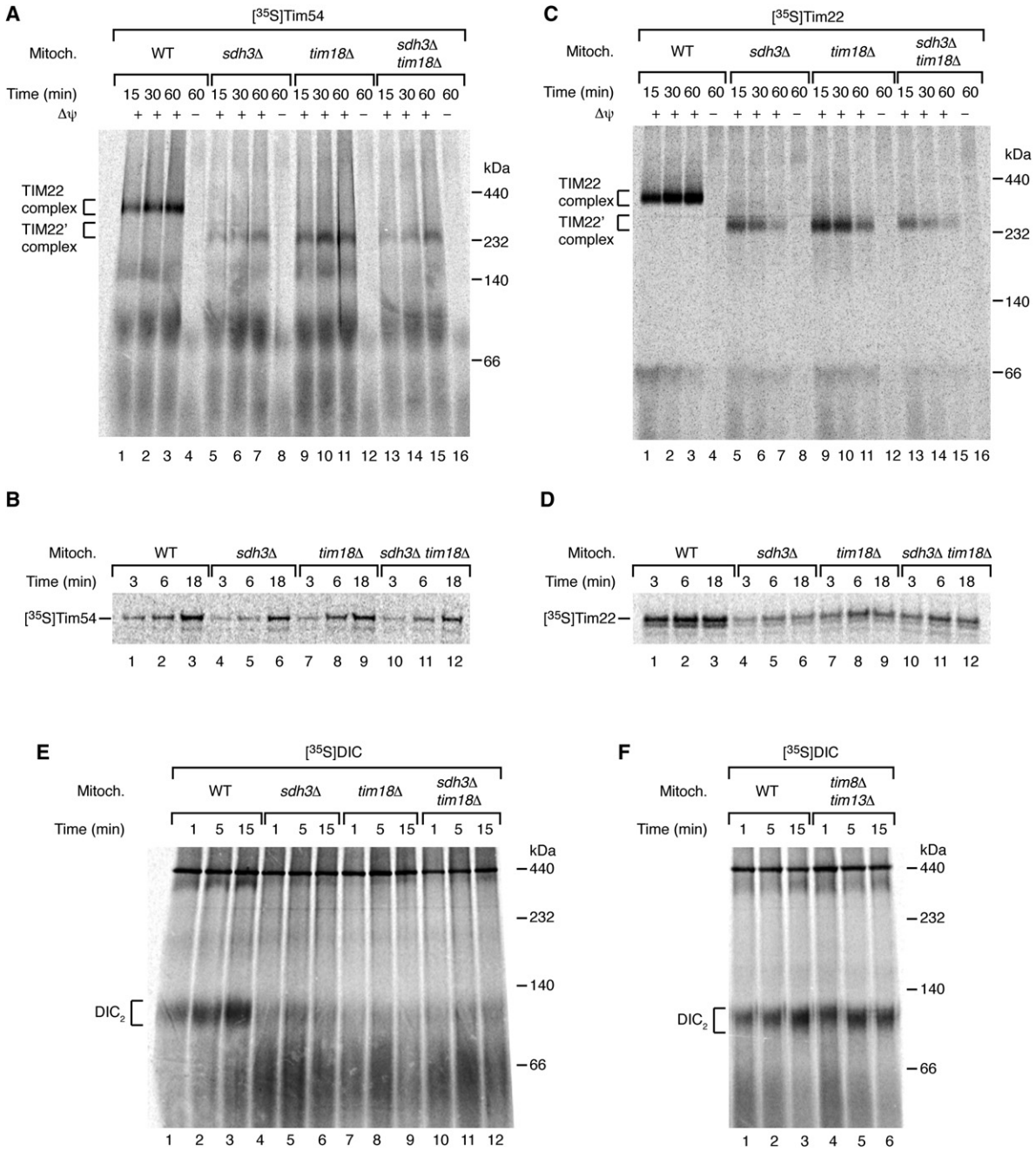

Figure 3. Mitochondria Lacking Sdh3 Are Impaired in Import of Tim22 and Carrier Precursors

(A) [^{35}S]Tim54 was incubated with isolated mitochondria. Mitochondria were treated with proteinase K, solubilized in digitonin, and analyzed by blue native electrophoresis and autoradiography. $\Delta\psi$, membrane potential.

(B) [^{35}S]Tim54 was imported into mitochondria, followed by treatment with proteinase K and separation by SDS-PAGE.

(C and D) [^{35}S]Tim22 was imported into mitochondria and analyzed as described in (A) and (B).

(E and F) [^{35}S]Dicarboxylate carrier (DIC) was imported into mitochondria as described in Supplemental Experimental Procedures (two-step import reaction) and analyzed by blue native electrophoresis. DIC_2, DIC dimer.

sdh3Δ, *tim18Δ*, and *sdh3Δ tim18Δ* mutant mitochondria. Tim54 assembled into the smaller TIM22′ complex of the mutant mitochondria. The yield of assembly was strongly decreased in *sdh3Δ* mitochondria compared to the yield of assembly into the TIM22 complex of wild-type mitochondria (Figure 3A). As reported by Wagner et al. (2008), the assembly of Tim54 was also impaired in *tim18Δ* mitochondria. The precursor of Tim54 is imported via the presequence translocase (Wagner et al.,

2008). Translocation of the Tim54 precursor to a protease-protected location was only mildly affected in the mutant mitochondria (Figure 3B), indicating that the lack of Sdh3 and Tim18 mainly affected the subsequent assembly into the translocase.

The assembly of radiolabeled Tim22 into the TIM22′ complex of *sdh3Δ*, *tim18Δ*, and *sdh3Δ tim18Δ* mutant mitochondria was also inhibited (Figure 3C). However, the precursor of Tim22 is imported via the carrier import pathway and thus is a substrate of the TIM22 complex itself. Import of the Tim22 precursor into the mutant mitochondria was indeed considerably impaired, as determined by translocation to a protease-protected location (Figure 3D). Thus, the lack of Sdh3 impairs the import of Tim22.

The metabolite carriers are main substrates of the TIM22 complex (Neupert and Herrmann, 2007; Chacinska et al., 2009). We analyzed the import of the dicarboxylate carrier via the TIM22 complex and the formation of its mature dimer. Import of the carrier protein was impaired in *sdh3Δ* and *tim18Δ* mutant mitochondria (Figure 3E), but not in control mutant mitochondria lacking the Tim8-Tim13 chaperone (Figure 3F). We conclude that mitochondria lacking Sdh3 are impaired in the import of substrates of the carrier pathway.

Sdh3 Is a Subunit of the TIM22 Complex and Interacts with Tim18

To determine if Sdh3 directly interacts with the TIM22 complex, we used a modified yeast strain that carried a cleavable protein A-tag fused to Tim18 (Rehling et al., 2003; Gebert et al., 2008). Mitochondria were isolated and lysed with digitonin, followed by IgG affinity chromatography to purify the TIM22 complex. In addition to the known subunits of the complex, Tim54, Tim22, and small Tim proteins, we copurified Sdh3 (Figure 4A). Sdh3 was the only additional component found in the copurification approach; neither further SDH subunits nor various control proteins of the mitochondrial inner or outer membranes were copurified. For comparison, we purified the SDH complex via protein A-tagged Sdh4 and isolated all four SDH proteins, Sdh1, Sdh2, Sdh3, and Sdh4, but none of the TIM proteins or other control proteins (Figure 4B). Thus, Sdh3 is associated with both the SDH complex and the TIM22 complex.

To show the presence of Sdh3 in two different protein complexes with wild-type mitochondria, we used blue native electrophoresis. Antibodies directed against Sdh3 decorated the 200 kDa SDH complex and a 300 kDa complex of the size of the TIM22 complex (Figure 4C). We radiolabeled the TIM22 complex by importing ^{35}S-labeled Tim18 or Tim22 into mitochondria and performed an antibody-shift analysis (Stojanovski et al., 2007). Antibodies against Sdh3, but neither anti-Sdh4 nor preimmune antibodies, quantitatively shifted the TIM22 complex on blue native gels (Figure 4D), demonstrating that Sdh3 is present in each TIM22 complex. We conclude that Sdh3 is a stoichiometric subunit of the mitochondrial TIM22 complex.

The assembly pathway of Tim18 involves a low-molecular-weight intermediate (Figure S2A) (Wagner et al., 2008). The intermediate was absent in *sdh3Δ* as well as *tim18Δ* mitochondria (Figure 2C, asterisk). In an antibody-shift analysis, antibodies directed against Sdh3 bound to the Tim18 intermediate but not to the Tim22 intermediate (Figure 4D), demonstrating that Sdh3 associates with the precursor of Tim18 on its assembly pathway. Tim12 and Tim54 are involved in the assembly of the mature TIM22 complex (Gebert et al., 2008; Lionaki et al., 2008; Wagner et al., 2008). We observed that the precursor of Tim18 accumulated in the intermediate form in *tim12-21* mutant mitochondria (Figure S2B), purified the intermediate by affinity chromatography, and confirmed the presence of Sdh3 by mass spectrometry (Figure S2C). Similarly, in *tim54Δ* mitochondria Tim18 accumulated in the intermediate form (Figure S2D). Taken together, Sdh3 is present in two different inner membrane protein complexes, the SDH complex and the TIM22 complex. Sdh3 associates with Tim18 on its biogenesis pathway and is crucial for the assembly of Tim18 into the TIM22 complex.

To directly demonstrate that Tim18 and Sdh4 recruit Sdh3 to two different complexes, we imported ^{35}S-labeled Sdh3 into wild-type mitochondria and coimported either chemical amounts of Tim18 or Sdh4. Whereas [^{35}S]Sdh3 assembled only with very low efficiency in the absence of the partner proteins, coimport of Tim18 directed Sdh3 into the TIM22 complex, and coimport of Sdh4 directed Sdh3 into the SDH complex (Figure 4E). Coimport of Tim18 or Sdh4 efficiently promoted assembly of [^{35}S]Sdh3 into the TIM22 complex of *tim18Δ* mitochondria (Figure 4E) or the SDH complex of *sdh4Δ* mitochondria (Figure S2E), respectively. To show that the lack of Tim18 affected the amount of endogenous Sdh3 in the TIM22 complex, we affinity-purified the complex with antibodies directed against Tim10 and observed that Sdh3 was lacking in the TIM22 complex purified from *tim18Δ* mitochondria (Figure S2F). Overexpression of neither Tim18 nor Sdh4 suppressed the growth defect of *sdh3Δ* cells (Figure S2G).

The yeast genome contains a predicted third member of the Sdh4/Tim18 family that is encoded by the ORF YLR164w (Kerscher et al., 2000; Koehler et al., 2000) and derived from the ancient genome duplication in *Saccharomyces cerevisiae* (Kellis et al., 2004). We detected the gene product in mitochondria and termed it Shh4 (for Sdh4 homolog). A fraction of Shh4 was copurified with tagged Sdh3 (Figure S2H), raising the possibility that Shh4 may be associated with SDH or the TIM22 complex. Deletion of *SHH4*, however, affected neither yeast growth nor the blue native mobility of SDH and TIM22 complex (Figure S2I). It is thus conceivable that three different Sdh3 complexes may exist in yeast mitochondria, each containing a different member of the Sdh4/Tim18 protein family (Sdh3-Shh4 being the putative third complex). The genome duplication also generated a homolog of Sdh3, encoded by the ORF YMR118c (Kellis et al., 2004) and termed Shh3 (for Sdh3 homolog). Similar to the situation with *SHH4*, a deletion of *SHH3* affected neither SDH nor TIM22 complex (Figure S2J). We conclude that neither Shh3 nor Shh4 are stoichiometric subunits of the SDH complex or the TIM22 complex.

To independently define the composition of the TIM22 complex, we analyzed the complex after stable isotope labeling with amino acids in cell culture (SILAC) (Ong et al., 2002). A yeast strain with protein A-tagged Tim18 and the corresponding wild-type strain were grown in synthetic medium. Proteins of the wild-type strain were metabolically labeled by supplementing the medium with the heavy amino acids [$^{13}C_6$]arginine and

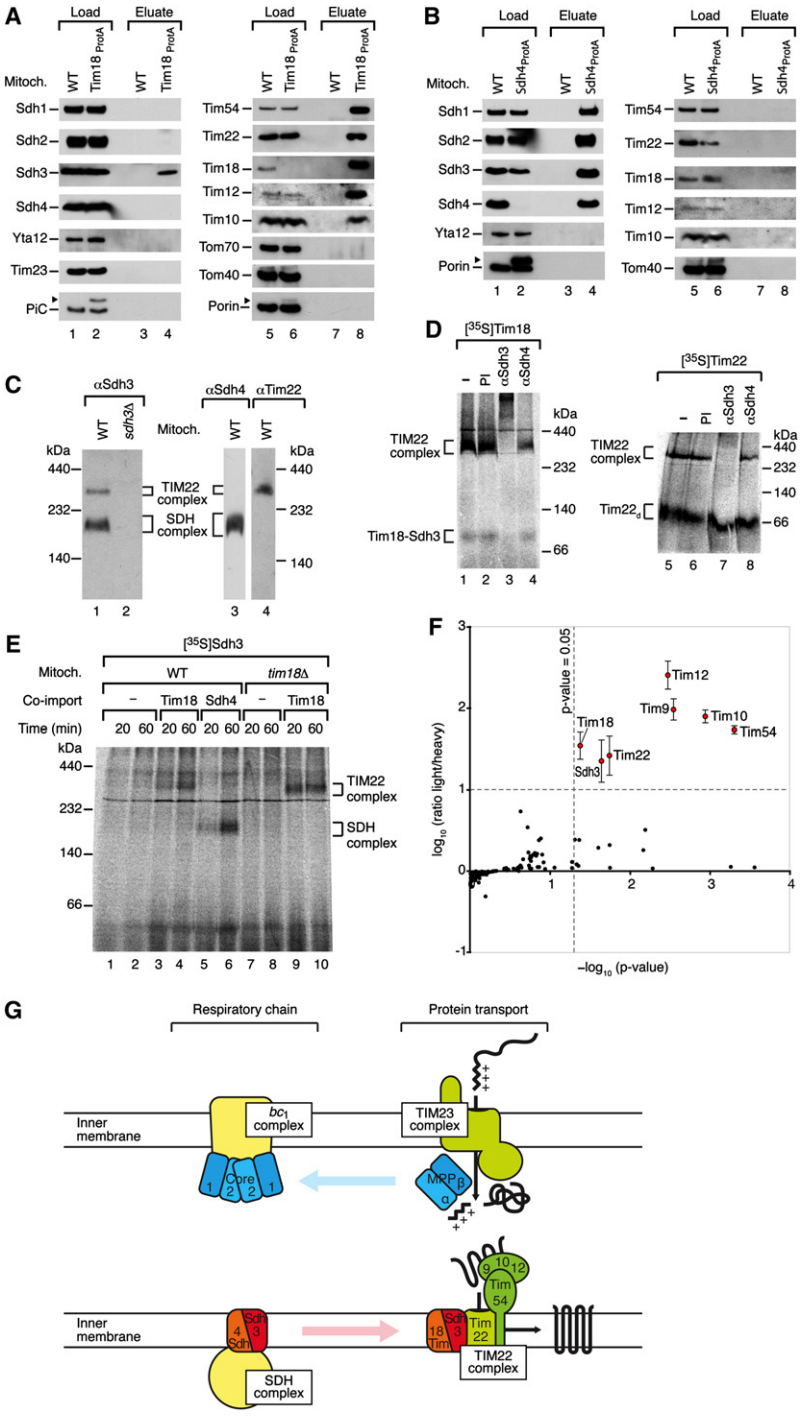

Figure 4. Sdh3 Is a Subunit of the TIM22 Complex

(A) Mitochondria isolated from wild-type (WT) and $Tim18_{ProtA}$ yeast strains were solubilized in digitonin, and protein complexes were isolated via the protein A-tag on Tim18. The samples were analyzed by SDS-PAGE and western blotting. Load, 10%; eluate, 100%; arrowheads, signal derived from $Tim18_{ProtA}$; PiC, phosphate carrier.

(B) Mitochondria isolated from wild-type and $Sdh4_{ProtA}$ strains were analyzed as described in (A). Arrowhead, signal derived from $Sdh4_{ProtA}$.

(C) Isolated mitochondria were lysed by digitonin and analyzed by blue native electrophoresis and western blotting.

(D) [^{35}S]Tim18 and Tim22 were imported into wild-type mitochondria. Mitochondria were solubilized in digitonin and incubated with the indicated antibodies. Protein complexes were analyzed by blue native electrophoresis and autoradiography. $Tim22_d$, Tim22 dimer; PI, pre-immune antibodies.

(E) [^{35}S]Sdh3 was imported into mitochondria in the presence of chemical amounts of Tim18 or Sdh4 as indicated. Digitonin-solubilized mitochondria were analyzed by blue native electrophoresis.

(F) Identification of Sdh3 as a component of the TIM22 complex by SILAC-based quantitative affinity purification-mass spectrometry (AP-MS). The TIM22 complex was purified from mitochondria containing tagged Tim18 (light amino acids) after mixing with untagged wild-type mitochondria that were labeled with heavy amino acids. The mean $\log_{10}$ light-over-heavy ratios (±SEM) of proteins quantified in the AP-MS experiments were plotted against the p values ($-\log_{10}$) (n = 3).

(G) Hypothetical model of the evolutionary relationship of respiratory complexes and the mitochondrial protein import machinery. See also Figure S2, Table S1, and Supplemental Experimental Procedures.

[$^{13}C_6$]lysine. Mitochondria were isolated from the two strains and mixed. After lysis with digitonin, the TIM22 complex was purified by affinity chromatography, and the eluates were analyzed by quantitative mass spectrometry. The ratios of the ^{12}C-containing (light) peptides derived from the $Tim18_{ProtA}$ strain over the ^{13}C-containing (heavy) peptides from the wild-type strain (light/heavy ratios) allowed us to assess the specificity of the copurification. All known subunits of the TIM22 complex were specifically copurified; Sdh3 was the only further protein that behaved like these subunits (Figure 4F; Table S1). Neither the other SDH subunits nor any other mitochondrial protein was significantly enriched in the eluate. In summary, the genetic, biochemical, and SILAC experiments together indicate that Sdh3 is a genuine subunit of the TIM22 complex.

DISCUSSION

We report that Sdh3 is a stoichiometric subunit not only of respiratory complex II (Oyedotun and Lemire, 2004; Sun et al., 2005) but also of the TIM22 complex. In complex II, Sdh3 and Sdh4 form a membrane-integrated heterodimer (Lemire and Oyedotun, 2002; Yankovskaya et al., 2003; Oyedotun and Lemire, 2004; Sun et al., 2005). We show that Sdh3 forms a complex with Tim18 during assembly of the TIM22 complex. Thus, the interaction with different yet homologous partner proteins, Sdh4 and Tim18, recruits Sdh3 into two inner membrane protein complexes, SDH and TIM22, with functions in bioenergetics and protein biogenesis, respectively. Modeling of the Sdh3-Tim18 complex based on the structure

of mitochondrial complex II suggests a high structural similarity of the Sdh3-Tim18 and Sdh3-Sdh4 complexes (Figures S2K–S2N) (Sun et al., 2005). In a yeast mutant lacking Sdh3, not only the levels of Sdh4 are dramatically reduced, but also the levels of Tim18, indicating that the complex formation with Sdh3 is crucial for the stability of Sdh4 as well as Tim18. Sdh3 is also required for the biogenesis of the two further integral membrane proteins of the TIM22 complex, Tim54 and Tim22, and the import of precursor proteins via the TIM22 complex. We conclude that Sdh3 is a genuine subunit of the TIM22 complex with functions in the biogenesis and assembly of the complex.

The identification of Sdh3 in the TIM22 complex provides insight into the evolution of the mitochondrial protein import machinery. In bacteria and mitochondria, respiratory complex II consists of two hydrophilic subunits (Sdh1 and Sdh2; termed SdhA and SdhB in bacteria) and two integral membrane proteins (Sdh3 and Sdh4; SdhC and SdhD) (Yankovskaya et al., 2003; Sun et al., 2005). Half of respiratory complex II, the membrane heterodimer Sdh3-Sdh4, was used as a building block for the TIM22 complex, leading to the evolution of the Sdh3-Tim18 dimer (Figure 4G). The channel-forming protein Tim22, like Tim23 and Tim17 of the presequence translocase, contains a region that is homologous to a signature motif of bacterial amino acid transporters (LivH) (Rassow et al., 1999; Dolezal et al., 2006). These observations suggest that the TIM22 complex may have evolved by combining two bacteria-derived modules, the membrane part of a respiratory complex and a protein derived from an amino acid transporter, followed by the eukaryotic addition of Tim54 and the Tim9-10-12 chaperone. Sdh3 is the only known integral membrane protein of the mitochondrial respiratory chain that is also located in a preprotein translocase. To date, only the peripheral subunits Core 1 and Core 2 of complex III (bc_1-complex) were found to be homologous and in some organisms identical to the subunits of the matrix processing peptidase (MPP), which cleaves preproteins imported via the TIM23 complex (Schulte et al., 1989; Dessi et al., 2000; Neupert and Herrmann, 2007). Bacterial complex III does not contain subunits corresponding to the eukaryotic Core 1 and 2 proteins, yet α-proteobacteria contain a proteolytically active homolog of MPP (Figure 4G). Thus, in the case of MPP, the proteolytic enzyme was present in bacteria and gave rise to additional subunits of a respiratory complex in eukaryotes. For Sdh3, the evolutionary development occurred in the opposite direction, from the respiratory complex (with integral subunits that were already present in bacteria) to the protein translocase in eukaryotes.

In summary, Sdh3 is present in at least two different protein complexes of the mitochondrial inner membrane and thus plays important roles in electron transfer and protein assembly.

EXPERIMENTAL PROCEDURES

Growth of Yeast, Isolation of Mitochondria, and Protein Import

S. cerevisiae strains were grown on YPS (2% sucrose), YPG (3% glycerol), or YPEG (2% ethanol and 2% glycerol) medium at 24°C–37°C. For overexpression assays, yeast cells were grown on YPEG plates. To induce the loss of mitochondrial DNA, yeast cells were grown on YPD (2% glucose) in the presence of 25 μg/ml ethidium bromide (three passages). For SILAC studies, the strain YPH500 (MATα, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, *lys2-801*) was used, and the *ARG8* locus was replaced by a *kanMX6* cassette. The resulting strain was auxotroph for arginine and lysine.

Mitochondria were isolated by differential centrifugation. For protein import into mitochondria, precursor proteins were synthesized in reticulocyte lysate in the presence of [^{35}S]methionine. Import experiments were performed in import buffer containing 2 mM ATP, 2 mM NADH, 5 mM creatine phosphate, and 0.1 mg/ml creatine kinase at 24°C–35°C (Stojanovski et al., 2007). Where indicated, mitochondria were treated with proteinase K. After the import reaction, mitochondria were either analyzed by SDS-PAGE or solubilized in 1% digitonin-containing buffer and separated by blue native electrophoresis, followed by digital autoradiography.

Protein Complex Purification and SILAC Analysis

Mitochondria isolated from yeast strains carrying a protein A-tag on Tim18 or Sdh4 were solubilized in digitonin-containing buffer. Protein complexes were purified via IgG affinity chromatography (Rehling et al., 2003) and eluted with either the tobacco etch virus protease or SDS. The eluates were analyzed by SDS-PAGE and western blotting.

For SILAC analysis, the strains YPH500*arg8Δ* ("wild-type") and Tim18$_{ProtA}$ were grown on nonfermentable SD medium. Proteins of the wild-type strain were labeled by substitution of [$^{12}C_6$]arginine and [$^{12}C_6$]lysine by their ^{13}C-isotopes (heavy amino acids). After isolation of mitochondria, wild-type and tagged mitochondria were mixed and solubilized prior to IgG affinity chromatography. Bound proteins were eluted with tobacco etch virus protease and analyzed by quantitative mass spectrometry.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, Supplemental Experimental Procedures, Supplemental References, and one table and can be found with this article online at doi:10.1016/j.molcel.2011.09.025.

ACKNOWLEDGMENTS

We are grateful to B. Lemire for antibodies and to T. Becker and C. Mehnert for discussion. This work was supported by the Deutsche Forschungsgemeinschaft, Gottfried Wilhelm Leibniz Program, Excellence Initiative of the German Federal and State Governments (EXC 294 BIOSS; GSC-4 Spemann Graduate School), Bundesministerium für Bildung und Forschung (Dynamo), Sonderforschungsbereich 746, Landesforschungspreis Baden-Württemberg, Trinationales Graduiertenkolleg GRK 1478 (D.A.S.), and an Alexander von Humboldt research fellowship (B.K.).

Received: April 15, 2011
Revised: August 18, 2011
Accepted: September 20, 2011
Published: December 8, 2011

REFERENCES

Chacinska, A., Koehler, C.M., Milenkovic, D., Lithgow, T., and Pfanner, N. (2009). Importing mitochondrial proteins: machineries and mechanisms. Cell *138*, 628–644.

Dessi, P., Rudhe, C., and Glaser, E. (2000). Studies on the topology of the protein import channel in relation to the plant mitochondrial processing peptidase integrated into the cytochrome bc_1 complex. Plant J. *24*, 637–644.

Dolezal, P., Likic, V., Tachezy, J., and Lithgow, T. (2006). Evolution of the molecular machines for protein import into mitochondria. Science *313*, 314–318.

Dunn, C.D., and Jensen, R.E. (2003). Suppression of a defect in mitochondrial protein import identifies cytosolic proteins required for viability of yeast cells lacking mitochondrial DNA. Genetics *165*, 35–45.

Dunn, C.D., Lee, M.S., Spencer, F.A., and Jensen, R.E. (2006). A genomewide screen for petite-negative yeast strains yields a new subunit of the i-AAA protease complex. Mol. Biol. Cell *17*, 213–226.

Gebert, N., Chacinska, A., Wagner, K., Guiard, B., Koehler, C.M., Rehling, P., Pfanner, N., and Wiedemann, N. (2008). Assembly of the three small Tim proteins precedes docking to the mitochondrial carrier translocase. EMBO Rep. *9*, 548–554.

Hwang, D.K., Claypool, S.M., Leuenberger, D., Tienson, H.L., and Koehler, C.M. (2007). Tim54p connects inner membrane assembly and proteolytic pathways in the mitochondrion. J. Cell Biol. *178*, 1161–1175.

Kellis, M., Birren, B.W., and Lander, E.S. (2004). Proof and evolutionary analysis of ancient genome duplication in the yeast *Saccharomyces cerevisiae*. Nature *428*, 617–624.

Kerscher, O., Sepuri, N.B., and Jensen, R.E. (2000). Tim18p is a new component of the Tim54p-Tim22p translocon in the mitochondrial inner membrane. Mol. Biol. Cell *11*, 103–116.

Koehler, C.M., Murphy, M.P., Bally, N.A., Leuenberger, D., Oppliger, W., Dolfini, L., Junne, T., Schatz, G., and Or, E. (2000). Tim18p, a new subunit of the TIM22 complex that mediates insertion of imported proteins into the yeast mitochondrial inner membrane. Mol. Cell. Biol. *20*, 1187–1193.

Lemire, B.D., and Oyedotun, K.S. (2002). The *Saccharomyces cerevisiae* mitochondrial succinate:ubiquinone oxidoreductase. Biochim. Biophys. Acta *1553*, 102–116.

Lionaki, E., de Marcos Lousa, C., Baud, C., Vougioukalaki, M., Panayotou, G., and Tokatlidis, K. (2008). The essential function of Tim12 in vivo is ensured by the assembly interactions of its C-terminal domain. J. Biol. Chem. *283*, 15747–15753.

Neupert, W., and Herrmann, J.M. (2007). Translocation of proteins into mitochondria. Annu. Rev. Biochem. *76*, 723–749.

Ong, S.E., Blagoev, B., Kratchmarova, I., Kristensen, D.B., Steen, H., Pandey, A., and Mann, M. (2002). Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol. Cell. Proteomics *1*, 376–386.

Oyedotun, K.S., and Lemire, B.D. (1999). The *Saccharomyces cerevisiae* succinate-ubiquinone reductase contains a stoichiometric amount of cytochrome b_{562}. FEBS Lett. *442*, 203–207.

Oyedotun, K.S., and Lemire, B.D. (2004). The quaternary structure of the Saccharomyces cerevisiae succinate dehydrogenase. Homology modeling, cofactor docking, and molecular dynamics simulation studies. J. Biol. Chem. *279*, 9424–9431.

Rassow, J., Dekker, P.J., van Wilpe, S., Meijer, M., and Soll, J. (1999). The preprotein translocase of the mitochondrial inner membrane: function and evolution. J. Mol. Biol. *286*, 105–120.

Rehling, P., Model, K., Brandner, K., Kovermann, P., Sickmann, A., Meyer, H.E., Kühlbrandt, W., Wagner, R., Truscott, K.N., and Pfanner, N. (2003). Protein insertion into the mitochondrial inner membrane by a twin-pore translocase. Science *299*, 1747–1751.

Schulte, U., Arretz, M., Schneider, H., Tropschug, M., Wachter, E., Neupert, W., and Weiss, H. (1989). A family of mitochondrial proteins involved in bioenergetICS and biogenesis. Nature *339*, 147–149.

Stojanovski, D., Pfanner, N., and Wiedemann, N. (2007). Import of proteins into mitochondria. Methods Cell Biol. *80*, 783–806.

Sun, F., Huo, X., Zhai, Y., Wang, A., Xu, J., Su, D., Bartlam, M., and Rao, Z. (2005). Crystal structure of mitochondrial respiratory membrane protein complex II. Cell *121*, 1043–1057.

Wagner, K., Gebert, N., Guiard, B., Brandner, K., Truscott, K.N., Wiedemann, N., Pfanner, N., and Rehling, P. (2008). The assembly pathway of the mitochondrial carrier translocase involves four preprotein translocases. Mol. Cell. Biol. *28*, 4251–4260.

Yankovskaya, V., Horsefield, R., Törnroth, S., Luna-Chavez, C., Miyoshi, H., Léger, C., Byrne, B., Cecchini, G., and Iwata, S. (2003). Architecture of succinate dehydrogenase and reactive oxygen species generation. Science *299*, 700–704.