

Role of mitochondrial inner membrane organizing system in protein biogenesis of the mitochondrial outer membrane

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ABSTRACT Mitochondria contain two membranes, the outer membrane and the inner membrane with folded cristae. The mitochondrial inner membrane organizing system (MINOS) is a large protein complex required for maintaining inner membrane architecture. MINOS interacts with both preprotein transport machineries of the outer membrane, the translocase of the outer membrane (TOM) and the sorting and assembly machinery (SAM). It is unknown, however, whether MINOS plays a role in the biogenesis of outer membrane proteins. We have dissected the interaction of MINOS with TOM and SAM and report that MINOS binds to both translocases independently. MINOS binds to the SAM complex via the conserved polypeptide transport-associated domain of Sam50. Mitochondria lacking mitofilin, the large core subunit of MINOS, are impaired in the biogenesis of β -barrel proteins of the outer membrane, whereas mutant mitochondria lacking any of the other five MINOS subunits import β -barrel proteins in a manner similar to wild-type mitochondria. We show that mitofilin is required at an early stage of β -barrel biogenesis that includes the initial translocation through the TOM complex. We conclude that MINOS interacts with TOM and SAM independently and that the core subunit mitofilin is involved in biogenesis of outer membrane β -barrel proteins.

Monitoring Editor

Benjamin S. Glick
University of Chicago

Received: Apr 16, 2012

Revised: Aug 2, 2012

Accepted: Aug 14, 2012

This article was published online ahead of print in MBoC in Press (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E12-04-0295>) on August 23, 2012.

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Abbreviations used: DiSC₃(5), dipropylthiadiazocarbocyanine iodide; ERMIONE, endoplasmic reticulum-mitochondria organizing network; Fcj1, formation of crista junction protein 1 (mitofilin); IgG, immunoglobulin G; MIA, mitochondrial intermembrane space assembly; MINOS, mitochondrial inner membrane organizing system; PMSF, phenylmethylsulfonyl fluoride; POTRA, polypeptide transport-associated; SAM, sorting and assembly machinery; TEV, tobacco etch virus; TOM, translocase of outer mitochondrial membrane.

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INTRODUCTION

Mitochondria consist of two membranes and two aqueous compartments, intermembrane space and matrix. The inner membrane is folded into tubular invaginations called cristae. Cristae junctions connect the cristae membranes with the remainder of the inner membrane, which is adjacent to the outer membrane and is called the inner boundary membrane (Frey and Mannella 2000; Mannella, 2006; Zick et al., 2009). Recent studies led to the identification of a large protein complex of the inner membrane that plays a crucial role in the maintenance of inner membrane architecture. The complex was termed mitochondrial inner membrane organizing system (MINOS), mitochondrial contact site complex, or mitochondrial organizing structure (Harner et al., 2011; Hoppins et al., 2011; Herrmann, 2011; von der Malsburg et al., 2011; Alkhaja et al., 2012; van der Laan et al., 2012). MINOS consists of six subunits that are all inner membrane proteins exposed to the intermembrane space. Two core proteins, mitofilin (formation of crista junction protein 1 [Fcj1]) and

Mio10 (Mcs10/Mos1/MINOS1), are essential for keeping the cristae membranes attached to the inner boundary membrane (John *et al.*, 2005; Rabl *et al.*, 2009; Mun *et al.*, 2010; Harner *et al.*, 2011; Head *et al.*, 2011; Hoppins *et al.*, 2011; von der Malsburg *et al.*, 2011; Alkhaja *et al.*, 2012; Zerbes *et al.*, 2012). The additional subunits, Aim5 (Mcs12), Aim13 (Mcs19/MINOS3), Aim37 (Mcs27), and Mio27 (Mcs29/Mos2), contribute to the integrity of the MINOS complex and maintenance of cristae architecture.

In addition to its role in inner membrane architecture, MINOS was found to interact with protein complexes of the outer mitochondrial membrane, including the two essential preprotein transport machineries (Xie *et al.*, 2007; Darshi *et al.*, 2011; Harner *et al.*, 2011; Hoppins *et al.*, 2011; von der Malsburg *et al.*, 2011; Alkhaja *et al.*, 2012; Körner *et al.*, 2012; Ott *et al.*, 2012; Zerbes *et al.*, 2012). The translocase of the outer mitochondrial membrane (TOM) forms the main entry gate for most nuclear-encoded mitochondrial precursor proteins, whereas the sorting and assembly machinery (SAM) mediates the insertion of β -barrel proteins into the mitochondrial outer membrane (Dolezal *et al.*, 2006; Neupert and Herrmann, 2007; Chacinska *et al.*, 2009; Dukanovic and Rapaport, 2011; Endo *et al.*, 2011; Becker *et al.*, 2012). These contact sites between inner and outer membranes are involved in the maintenance of cristae morphology (Körner *et al.*, 2012; Ott *et al.*, 2012). In addition, it was shown that mitofilin/Fcj1 supports the transport of small precursor proteins into the intermembrane space (von der Malsburg *et al.*, 2011). Mitofilin contains a large intermembrane space domain that interacts with the TOM complex and the receptor Mia40 of the mitochondrial intermembrane space assembly (MIA) machinery (Chacinska *et al.*, 2004; Mesecke *et al.*, 2005; Dabir *et al.*, 2007; Grumbt *et al.*, 2007; Stojanovski *et al.*, 2008; Banci *et al.*, 2009; Kawano *et al.*, 2009; Koehler and Tienison, 2009; Bien *et al.*, 2010). The transient interaction of mitofilin with Mia40 helps to position this intermembrane space receptor in the vicinity of the TOM complex, and precursor proteins passing through the TOM channel can therefore be immediately captured by Mia40 (von der Malsburg *et al.*, 2011).

The mitochondrial outer membrane contains two major protein types: proteins with α -helical transmembrane segments and β -barrel

proteins. The precursors of β -barrel proteins are initially imported via the TOM complex and translocated to the intermembrane space (Model *et al.*, 2001; Paschen *et al.*, 2003; Wiedemann *et al.*, 2003, 2004; Mihara, 2003). Chaperone complexes of the small TIM-type help in transfer of the hydrophobic precursors to the SAM complex that mediates insertion of the proteins into the outer membrane (Paschen *et al.*, 2003, 2005; Wiedemann *et al.*, 2003, 2004; Gentile *et al.*, 2004; Hoppins and Nargang, 2004; Habib *et al.*, 2005; Chan and Lithgow, 2008; Kutik *et al.*, 2008). For α -helical precursor proteins, several import pathways have been described that can involve TOM receptors, SAM, and other outer membrane proteins (Stojanovski *et al.*, 2007a; Becker *et al.*, 2008, 2009, 2011; Hulett *et al.*, 2008; Kemper *et al.*, 2008; Popov-Čeleketić *et al.*, 2008; Thornton *et al.*, 2010; Dukanovic and Rapaport, 2011; Papić *et al.*, 2011; Dimmer *et al.*, 2012). Because MINOS interacts with both outer membrane translocases, it may potentially be connected to protein import into the outer membrane. However, different views on the relation of MINOS to the biogenesis of outer membrane proteins have been reported (Darshi *et al.*, 2011; Körner *et al.*, 2012).

For this report, we analyzed the interaction of MINOS with TOM and SAM. We show that MINOS binds to TOM and SAM in an independent manner. Mutant mitochondria lacking mitofilin/Fcj1 were impaired in the biogenesis of β -barrel proteins. Mitofilin is involved in an early stage of β -barrel import that includes the translocation of precursor proteins through the TOM complex. We conclude that mitofilin not only promotes the import of small proteins into the intermembrane space (von der Malsburg *et al.*, 2011) but also the biogenesis of β -barrel proteins of the outer membrane.

RESULTS

Differential copurification of TOM and SAM with MINOS subunits

We asked whether MINOS interacts with TOM and SAM simultaneously or whether distinct outer membrane contacts exist. In a first approach, we performed pulldown experiments with Protein A-tagged MINOS components. We used a *Saccharomyces cerevisiae* strain expressing mitofilin/Fcj1 with a C-terminal protein A tag (von der Malsburg *et al.*, 2011) and generated a yeast strain that expressed Mio27 with a C-terminal protein A tag. The protein A tags were attached to the MINOS components via a linker containing a tobacco etch virus (TEV) protease cleavage site. Extracts of the yeast cells were prepared using the nonionic detergent digitonin and subjected to immunoglobulin G (IgG) affinity chromatography. Bound proteins were eluted by cleavage with TEV protease. Fcj1_{ProtA} and Mio27_{ProtA} copurified the other five MINOS subunits with comparable efficiency (Figure 1, lanes 5 and 6), whereas control proteins of the inner and outer membranes were not found in the eluate (Figure 1, lanes 5, 6, 11, and 12). In addition to the MINOS subunits, Fcj1_{ProtA} copurified TOM and SAM subunits (Figure 1, lane 11; the receptor Tom70 is only loosely associated with the yeast TOM complex and is therefore copurified in minor amounts; Meisinger *et al.*, 2001). In contrast, Mio27_{ProtA} neither copurified the channel-forming protein Tom40 nor the receptors Tom20, Tom22, and Tom70

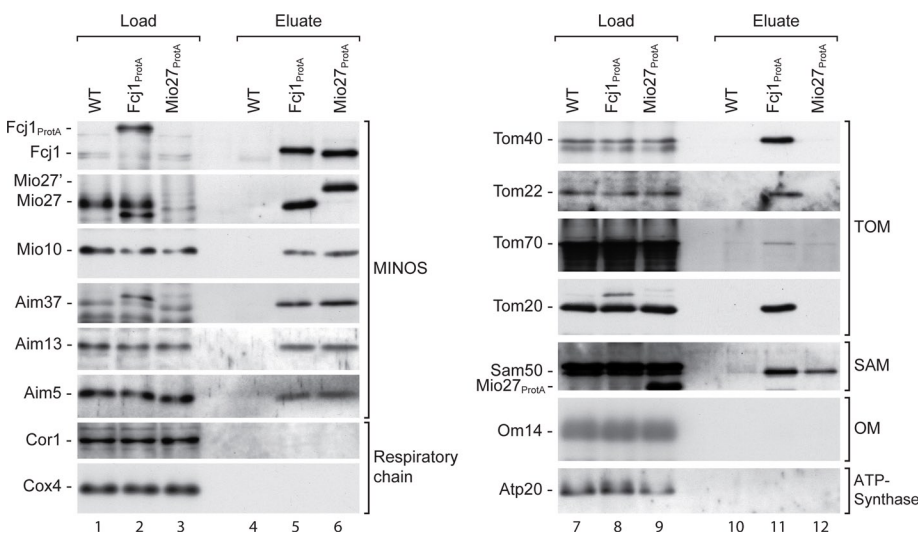


FIGURE 1: MINOS interacts with outer membrane protein complexes. Whole-cell digitonin extracts from wild-type (WT) cells and cells expressing protein A fusion constructs (Fcj1_{ProtA} and Mio27_{ProtA}) were subjected to IgG affinity chromatography, elution with TEV protease, and analysis by SDS-PAGE and immunoblotting. Load, 1.5%; elution, 100%. Mio27_{ProtA}, TEV-cleaved form of Mio27_{ProtA}; OM, outer mitochondrial membrane.

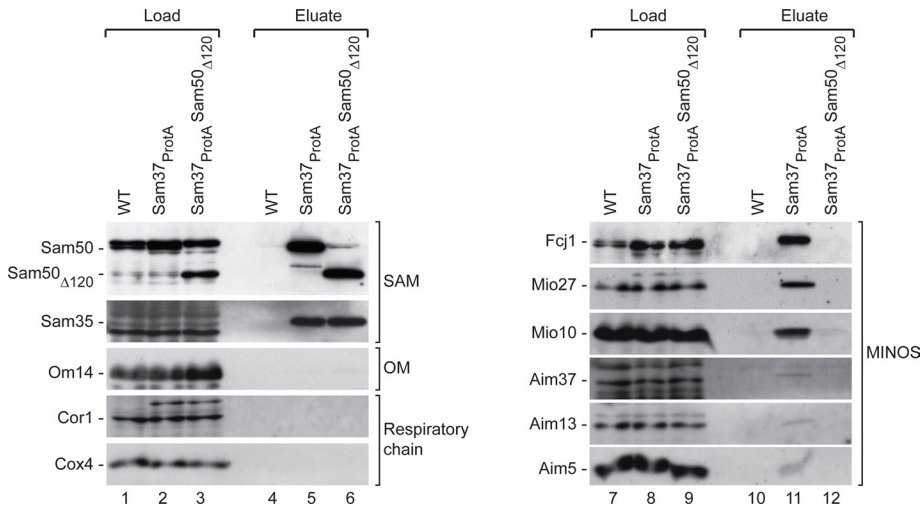


FIGURE 2: Interaction between MINOS and SAM depends on the POTRA domain of Sam50. Whole-cell powder from wild-type (WT), Sam37^{ProtA}, and Sam37^{ProtA} Sam50 Δ ₁₂₀ cells was solubilized in digitonin-containing buffer, subjected to IgG affinity chromatography, and analyzed by SDS-PAGE and immunoblotting. Load, 1%; elution, 100%. OM, outer mitochondrial membrane.

(Figure 1, lane 12), demonstrating that the TOM complex was not pulled down with tagged Mio27. However, Mio27^{ProtA} copurified Sam50 (Tob55), the core component of the SAM complex, though with a reduced efficiency compared with the copurification with Fcj1^{ProtA} (Figure 1, lanes 11 and 12). These findings raised the possibility that binding of TOM and SAM to MINOS is not coupled but can be separated.

Requirement of the polypeptide transport-associated domain of Sam50 for MINOS–SAM interaction but not for MINOS–TOM interaction

To obtain direct evidence for an independent interaction of MINOS with the outer membrane translocases, we sought determinants that are required for formation of the interactions. The SAM

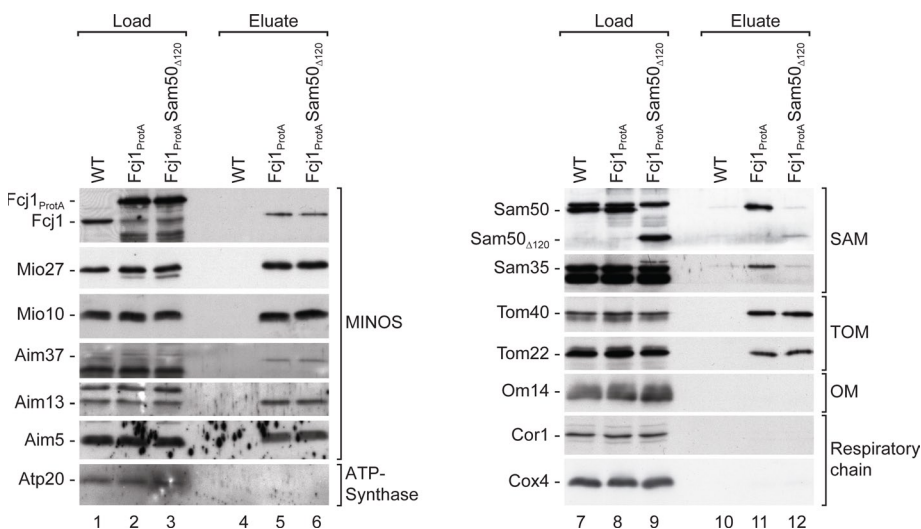


FIGURE 3: MINOS independently interacts with outer membrane TOM and SAM complexes. Whole-cell digitonin extracts from wild-type (WT), Fcj1^{ProtA}, and Fcj1^{ProtA} Sam50 Δ ₁₂₀ cells were subjected to IgG affinity chromatography and analyzed by SDS-PAGE and immunoblotting. Load, 1.5%; elution, 100%. OM, outer mitochondrial membrane.

complex contains one large hydrophilic domain that is exposed to the intermembrane space, the polypeptide transport-associated (POTRA) domain at the N-terminus of Sam50 (Kozjak *et al.*, 2003; Paschen *et al.*, 2003; Sánchez-Pulido *et al.*, 2003; Gentle *et al.*, 2004; Habib *et al.*, 2007; Knowles *et al.*, 2008; Kutik *et al.*, 2008; Stroud *et al.*, 2011a), whereas Sam35 and Sam37 expose domains to the cytosolic side (Wiedemann *et al.*, 2003; Ishikawa *et al.*, 2004; Milenkovic *et al.*, 2004; Waizenegger *et al.*, 2004; Chan and Lithgow, 2008; Kutik *et al.*, 2008). We used a yeast strain that expressed Sam37 with a protein A tag (Kozjak *et al.*, 2003). From a digitonin extract of the cells, tagged Sam37 pulled down additional SAM proteins, Sam35 and Sam50, as expected (Figure 2, lane 5; Kozjak *et al.*, 2003), but also the subunits of MINOS (Figure 2, lane 11; Aim5, Aim13, and Aim37 were copurified with lower yield than Fcj1, Mio10, and Mio27). Control proteins of the outer and inner membranes were not copurified (Figure 2, lane 5). We generated a Sam37^{ProtA} strain, in which the N-terminal 120 residues of Sam50, including the entire POTRA domain, were deleted (Sam50 Δ ₁₂₀; Kutik *et al.*, 2008; Stroud *et al.*, 2011a). Copurification of the subunits of the SAM complex was not affected by the lack of the POTRA domain (Figure 2, lane 6; Habib *et al.*, 2007; Stroud *et al.*, 2011a). However, the pulldown of MINOS subunits with tagged Sam37 was strongly inhibited when the POTRA domain of Sam50 was lacking (Figure 2, lane 12). These results indicate that the POTRA domain is required for the interaction of the SAM complex with MINOS.

To address whether the POTRA domain was required for the interaction of TOM with MINOS, we generated an Fcj1^{ProtA} strain in which the POTRA domain of Sam50 was deleted (Figure 3). Tagged Fcj1 pulled down the other five MINOS subunits independently of the presence or absence of the POTRA domain (Figure 3, lanes 5 and 6). The interaction of Fcj1 with Sam50 and Sam35 was strongly inhibited by the lack of the POTRA domain (Figure 3, lane 12; the steady-state levels of Sam50 and Sam35 were not affected; Figure 3, lanes 8 and 9). However, Tom22 and Tom40 were efficiently copurified with tagged Fcj1 and did not require the presence of the Sam50 POTRA domain (Figure 3, lanes 11 and 12). The mitochondrial ultrastructure analyzed by electron microscopy was not altered when mitofilin/Fcj1 carried a protein A tag and was only mildly affected by the lack of the Sam50 POTRA domain (Supplemental Figure S1), indicating that the POTRA-mediated MINOS–SAM interaction is not strictly essential for maintaining the architecture of the mitochondrial inner membrane.

We conclude that mitofilin/Fcj1 can bind TOM despite an impaired interaction between mitofilin/Fcj1 and SAM. In Figure 1 we show that tagged Mio27 pulled down

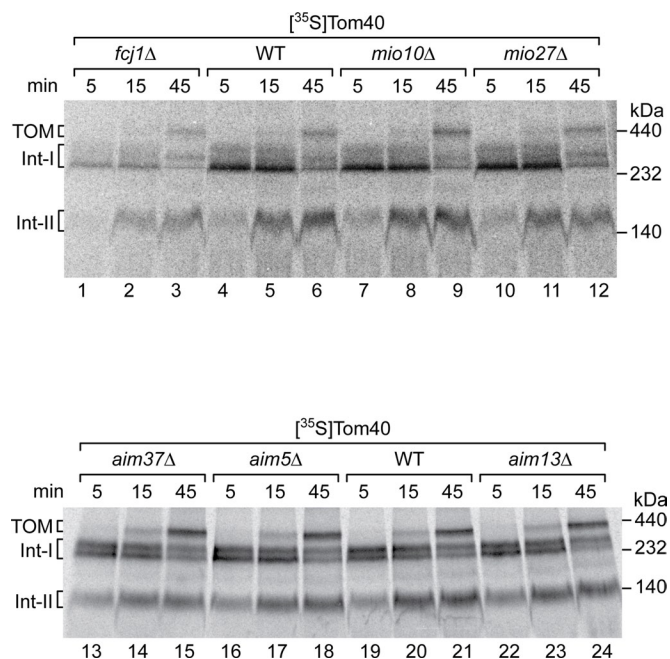


FIGURE 4: Deletion of *FCJ1*, but not of other MINOS components, leads to impaired Tom40 biogenesis. [³⁵S]-labeled Tom40 was imported into mitochondria isolated from wild-type (WT), *fcj1Δ*, *mio10Δ*, *mio27Δ*, *aim37Δ*, *aim5Δ*, and *aim13Δ* cells. Mitochondria were solubilized with digitonin and subjected to blue native electrophoresis and digital autoradiography. Int-I, precursor-SAM assembly intermediate I; Int-II, assembly intermediate II.

SAM but not the TOM complex, indicating that the MINOS-SAM interaction does not require interaction with the TOM complex. Taken together, these findings demonstrate that TOM and SAM independently interact with components of the MINOS machinery of the inner membrane.

Biogenesis of outer membrane proteins in MINOS mutant mitochondria

Biogenesis of mitochondrial β -barrel proteins requires both TOM and SAM (Endo and Yamano, 2009; Dukanovic and Rapaport, 2011; Becker et al., 2012). To study whether MINOS was involved in outer membrane protein biogenesis, we used the radiolabeled precursor of Tom40 as a model substrate. For this precursor, three assembly stages can be resolved by blue native electrophoresis of digitonin-lysed mitochondria (Model et al., 2001; Paschen et al., 2003; Wiedemann et al., 2003; Ishikawa et al., 2004; Chan and Lithgow, 2008; Dukanovic et al., 2009). On incubation with isolated mitochondria, Tom40 forms intermediate I, which represents interaction of the precursor with the SAM complex (Model et al., 2001; Wiedemann et al., 2003; Becker et al., 2010). Subsequently, the precursor forms a smaller intermediate II before it is assembled into the mature TOM complex (Figure 4, lanes 4–6). We isolated mitochondria from single-deletion yeast strains of the six MINOS subunits and imported the precursor of Tom40. Most of the mutant mitochondria imported and assembled Tom40 in a manner similar to wild-type mitochondria (Figure 4, lanes 4–24). The only exception was mitochondria lacking mitofilin/Fcj1, which were considerably impaired in all three assembly stages of Tom40 (Figure 4, lanes 1–3).

We analyzed the steady-state levels of TOM, SAM, and TIM proteins and did not observe any substantial differences between *fcj1Δ* and wild-type mitochondria that would explain the defect in Tom40

biogenesis (Figure S2A). Moreover, the stability of neither the TOM complex nor the SAM complex, as analyzed by blue native electrophoresis, was affected by the lack of mitofilin/Fcj1 (Figure S2B). The biogenesis of other β -barrel proteins of the outer mitochondrial membrane, porin and Mdm10, was analyzed by monitoring assembly of the radiolabeled precursors. These precursors are also imported via the TOM and SAM complexes but with faster kinetics than Tom40, and the wild-type precursors therefore do not form stable SAM intermediates in considerable amounts (Wiedemann et al., 2003; Kutik et al., 2008; Stroud et al., 2011a). Biogenesis of porin and Mdm10 was retarded in *fcj1Δ* mitochondria but not in *mio10Δ* mitochondria (Figure S2, C and D), supporting the view that mitofilin/Fcj1, but not Mio10, is involved in the efficient import of outer membrane β -barrel proteins.

We performed several control experiments to exclude possible indirect effects. 1) Major alterations of mitochondrial phospholipid levels, such as lack of cardiolipin, can impair the biogenesis of outer membrane proteins (Gebert et al., 2009). We compared the phospholipid composition of mitochondria from *fcj1Δ* and wild-type yeast. In addition, mitochondria lacking Mio10 were analyzed. Figure S3 shows that the phospholipid composition was not substantially changed by the lack of the central MINOS components mitofilin/Fcj1 or Mio10. 2) We generated a yeast strain in which *FCJ1* was expressed under the control of a galactose-inducible promoter. On shift of the cells to glucose-containing medium, the levels of Fcj1 were decreased (the levels of TOM, SAM, and TIM components, as well as the stability of TOM and SAM complexes, were not affected; Figure S4, A and B). We selected conditions under which Fcj1 was strongly depleted (Figure S4A), yet the inner membrane potential $\Delta\psi$ was comparable with that of wild-type mitochondria (Figure 5A). For *fcj1Δ* mitochondria, it has been reported that $\Delta\psi$ is partially decreased, and the $\Delta\psi$ -dependent import of proteins into or across the inner membrane is therefore also partially reduced (von der Malsburg et al., 2011). In contrast, the Fcj1-depleted mitochondria imported the matrix protein F₁-ATPase subunit β and the inner membrane proteins cytochrome *c*₁ and ADP/ATP carrier with an efficiency close to that of wild-type mitochondria (Figure 5, B and C). Though the import of mitochondrial outer membrane proteins does not require the inner membrane potential (Chacinska et al., 2009), use of the Fcj1-depleted mitochondria offered the opportunity to minimize pleiotropic effects. The assembly of the precursor of Tom40 was impaired in Fcj1-depleted mitochondria (Figure 5D), supporting the view of a specific role of mitofilin/Fcj1 in this process.

We conclude that lack of mitofilin/Fcj1 impairs the biogenesis pathway of β -barrel precursors, whereas other MINOS components are not required for β -barrel assembly.

Lack of mitofilin/Fcj1 impairs biogenesis of Tom40 at a stage before the SAM complex

At which stage of Tom40 assembly is mitofilin/Fcj1 involved? Because the formation of the SAM-bound state (assembly intermediate I on native gels) is strongly impaired in *fcj1Δ* mitochondria, either the SAM complex itself or a step leading to the SAM complex is compromised. We imported the radiolabeled precursor of Tom22 that uses TOM receptors and each subunit of the SAM complex before its assembly into the TOM complex (Keil and Pfanner, 1993; Meisinger et al., 2004; Stojanovski et al., 2007a; Dukanovic et al., 2009; Thornton et al., 2010). Assembly of radiolabeled Tom22 into the TOM complex occurred with similar efficiency in *fcj1Δ* mitochondria, *mio10Δ* mitochondria, and wild-type mitochondria (Figure S5A). Thus the SAM complex and TOM receptors are functional in

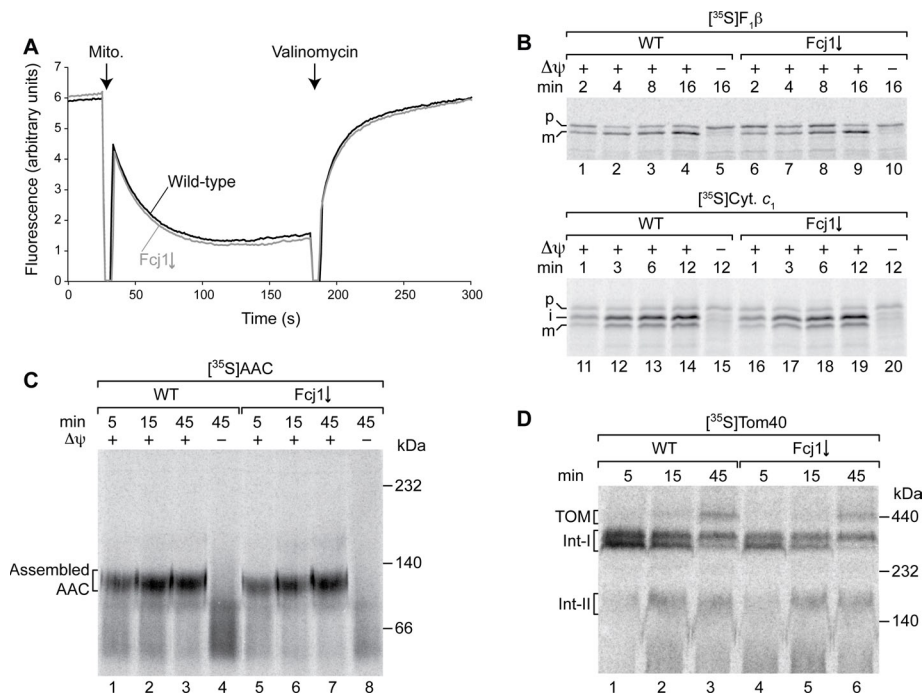


FIGURE 5: Biogenesis of Tom40 is impaired upon depletion of Fcj1. (A) Fcj1 \downarrow (YPH499 *fcj1::kanMX6, PGAL1-FCJ1*) and wild-type control cells were precultured in the presence of 2% galactose. Subsequently, expression of *PGAL1-FCJ1* was inhibited with 1% glucose, mitochondria were isolated, and the mitochondrial membrane potential was assessed using the potential-sensitive dye DiSC₃(5). (B) The [³⁵S]-labeled precursors of F₁-ATPase subunit β (F₁ β) and cytochrome c₁ (Cyt. c₁) were imported into isolated mitochondria for the indicated time periods. After proteinase K treatment to remove nonimported precursors, samples were analyzed by SDS-PAGE and digital autoradiography. p, precursor; i, intermediate; m, mature. (C) [³⁵S]ADP/ATP carrier (AAC) or (D) [³⁵S]Tom40 was imported into isolated mitochondria as indicated and analyzed by blue native electrophoresis and digital autoradiography. Int-I, precursor-SAM assembly intermediate I; Int-II, assembly intermediate II.

fcj1 Δ mitochondria (the precursor of Tom22 is not translocated through the TOM channel to the intermembrane space but is directly inserted into the outer membrane by the SAM complex; Stojanovski *et al.*, 2007a; Thornton *et al.*, 2010). Import and assembly of the precursor of Tom5 into the TOM complex was also not affected by the lack of Fcj1 or Mio10 (Figure S5B).

Because the POTRA domain is required for the MINOS-SAM interaction, we compared Tom40 assembly in Sam50 Δ_{120} mitochondria with Tom40 assembly in *fcj1* Δ mitochondria. It has been reported that lack of the POTRA domain only mildly affects the biogenesis of radiochemical amounts of Tom40 (Kutik *et al.*, 2008; Stroud *et al.*, 2011a), and *fcj1* Δ mitochondria therefore apparently show a much stronger defect in Tom40 biogenesis. To directly compare this with the pulldown experiments that were dependent on the presence of the POTRA domain (Figure 2), we studied the import of Tom40 into Sam37^{ProtA} mitochondria lacking the POTRA domain of Sam50. Tom40 assembly was not inhibited in the POTRA-deficient mutant mitochondria (Figure 6A). Because the POTRA domain is required for a stable MINOS-SAM interaction, the biogenesis pathway of Tom40 is not inhibited when the MINOS-SAM interaction is disturbed. Taken together with the full activity of the SAM complex in the assembly of Tom22, these results suggest that the Tom40 assembly defect in *fcj1* Δ mitochondria may occur at a step preceding formation of the SAM-precursor intermediate.

The early steps in Tom40 biogenesis involve initial translocation across the outer membrane to the intermembrane space and binding to the small TIM chaperones (Model *et al.*, 2001; Mihara, 2003;

Wiedemann *et al.*, 2003, 2004; Hoppins and Nargang, 2004). These early steps do not involve blue native-stable intermediates and thus cannot be directly visualized by native gel electrophoresis (Wiedemann *et al.*, 2004; Rao *et al.*, 2012). After a short import time, the precursor of Tom40 was associated with tagged Fcj1 (Figure S5C), suggesting an involvement of mitofilin/Fcj1 at an early import stage. The translocation of the Tom40 precursor across the outer membrane can be assessed by its protection against externally added protease (Wiedemann *et al.*, 2003, 2004; Paschen *et al.*, 2003; Chan and Lithgow, 2008; Dukanovic *et al.*, 2009). We imported radiolabeled Tom40 precursor into isolated mitochondria. The efficiency of Tom40 translocation to a protease-protected location was significantly reduced in *fcj1* Δ mitochondria compared with wild-type mitochondria (Figure 6, B and C), indicating that this initial import step of Tom40 is affected by the lack of mitofilin/Fcj1.

Taken together, mitochondria lacking mitofilin/Fcj1 are impaired in an early step of Tom40 biogenesis that precedes the SAM complex and includes the initial translocation across the outer membrane.

DISCUSSION

We report a new function for mitofilin/Fcj1 and the MINOS machinery of the mitochondrial inner membrane. This membrane organizing system is not only involved in the

maintenance of mitochondrial cristae morphology (Harner *et al.*, 2011; Hoppins *et al.*, 2011; von der Malsburg *et al.*, 2011; Alkhaja *et al.*, 2012; Körner *et al.*, 2012; Ott *et al.*, 2012; Zerbes *et al.*, 2012) and protein import via the MIA pathway into the intermembrane space (von der Malsburg *et al.*, 2011), but also in the biogenesis of outer membrane proteins with β -barrel topology.

Both protein translocases of the outer membrane, TOM and SAM, independently bind to MINOS. 1) We observed for the SAM complex that the conserved POTRA domain on the intermembrane space side of Sam50 is required for the stable interaction with MINOS, whereas the binding of TOM to MINOS occurs independently of the POTRA domain. 2) The TOM complex is efficiently pulled down only by tagged mitofilin/Fcj1 but not by other tagged subunits of MINOS, such as Mio27 and Aim5, although these components copurify all other MINOS subunits (von der Malsburg *et al.*, 2011; this study). In contrast, the SAM complex is not only copurified with mitofilin/Fcj1 but also with several other MINOS components (Xie *et al.*, 2007; Darshi *et al.*, 2011; Harner *et al.*, 2011; Alkhaja *et al.*, 2012; this study). Thus MINOS can be found in association with the SAM complex independently of the MINOS-TOM interaction. 3) When the MINOS complex is fully or partially disrupted by deletion of *MIO10*, *AIM5*, or *AIM13*, the interaction of mitofilin/Fcj1 with the TOM complex is not disturbed but occurs with wild-type efficiency (von der Malsburg *et al.*, 2011), demonstrating that an intact MINOS complex is not required for the mitofilin/Fcj1-TOM interaction. Taken together, these data indicate two distinct forms of contact sites between outer membrane translocases and the inner

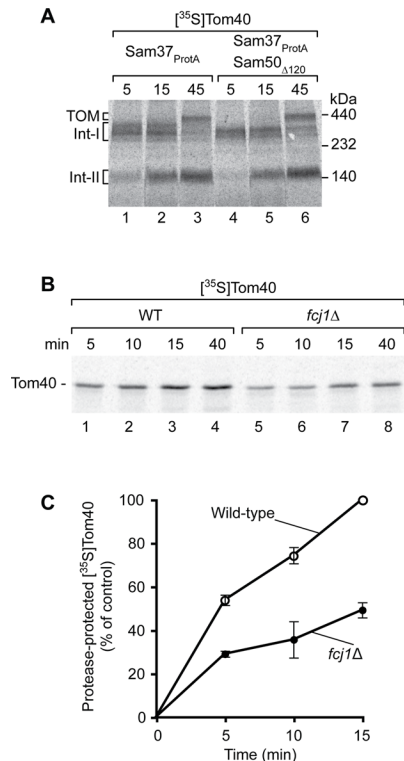


FIGURE 6: Mitofilin/Fcj1 is required at an early stage of Tom40 biogenesis. (A) [³⁵S]-labeled Tom40 was imported into Sam37_{ProtA} and Sam37_{ProtA} Sam50 Δ ₁₂₀ mitochondria; this was followed by solubilization in digitonin buffer, blue native electrophoresis, and autoradiography. Int-I, precursor-SAM assembly intermediate I; Int-II, assembly intermediate II. (B) Radiolabeled Tom40 was incubated with wild-type (WT) and *fcj1Δ* mitochondria. Nonimported precursor was removed by proteinase K treatment, and mitochondria were subjected to SDS-PAGE and digital autoradiography. (C) Tom40 import experiments were performed as described in (B) and quantified. Data are represented as mean \pm SE of the mean ($n = 3$), with the exception of the 10-min time point ($n = 2$; error bar represents range). The amount of protease-protected [³⁵S]Tom40 after 15-min import into wild-type mitochondria was set to 100% (control).

membrane organizing system are formed: a MINOS-SAM contact that requires the POTRA domain and likely includes the entire MINOS complex, and a mitofilin/Fcj1-TOM contact that does not depend on the other MINOS components.

We analyzed mitochondria that were isolated from yeast single-deletion mutants of each of the six MINOS genes for the biogenesis of outer membrane proteins. Remarkably, only mitochondria lacking mitofilin/Fcj1 were impaired in the assembly pathway of β -barrel proteins, as assessed with the model substrate Tom40. All other mutant mitochondria imported Tom40 with wild-type efficiency. Since *fcj1Δ* and *mio10Δ* mutants show a comparably strong degree of morphological alteration of the mitochondrial inner membrane (Harner et al., 2011; Hoppins et al., 2011; von der Malsburg et al., 2011; Alkhaja et al., 2012), it can be excluded that the β -barrel assembly defect is indirectly caused by the morphological defect. Moreover, we observed that the phospholipid profiles of *fcj1Δ* and *mio10Δ* mitochondria are similar to those of wild-type mitochondria. We dissected the biogenesis pathway of Tom40 into distinct stages and observed that the lack of mitofilin/Fcj1 affected an early import step that includes translocation of the Tom40 precursor through the TOM complex to the intermembrane

space. The activity of the SAM complex itself was not compromised in *fcj1Δ* mitochondria, since the SAM-dependent precursor of Tom22 was efficiently assembled in the mutant mitochondria and the POTRA-dependent MINOS-SAM interaction was not required for Tom40 assembly.

These findings suggest a model in which mitofilin/Fcj1 is present in at least two pools. On the one hand, mitofilin/Fcj1 is a subunit of the MINOS complex. This complex is crucial for maintenance of inner membrane morphology and also mediates the interaction with the SAM complex. On the other hand, a fraction of mitofilin/Fcj1 molecules are also functional without the other MINOS components. These mitofilin/Fcj1 molecules interact with the TOM complex and are involved in the biogenesis of β -barrel proteins of the outer membrane. Interestingly, the import of intermembrane space proteins via the MIA pathway is only impaired in *fcj1Δ* mitochondria but not in deletion mutants of other MINOS components (von der Malsburg et al., 2011), supporting the view that the roles of mitofilin/Fcj1 in promoting protein biogenesis via the β -barrel and MIA pathways are performed by the mitofilin/Fcj1 pool, which does not depend on an intact MINOS complex. It has been suggested MINOS, SAM, and TOM are part of a large endoplasmic reticulum-mitochondria organizing network (ERMIONE) that is involved in controlling mitochondrial architecture and biogenesis (van der Laan et al., 2012). The findings reported here support the view that ERMIONE functions as a dynamic network (Zerbes et al., 2012).

MATERIALS AND METHODS

Yeast strains

S. cerevisiae strains used in this study are derivatives of YPH499 (MATa, *ade2-101*, *his3-Δ200*, *leu2-Δ1*, *ura3-52*, *trp1-Δ63*, *lys2-801*; Sikorski and Hieter, 1989). YPH499 strains *fcj1Δ*, *mio10Δ*, *mio27Δ*, *aim5Δ*, *aim13Δ*, and *aim37Δ* were generated by homologous recombination using *kanMX4* cassettes amplified from genomic DNA from strains *fcj1Δ* (BY4741), *mio10Δ* (BY4741), *mio27Δ* (BY4741), *aim5Δ* (BY4741), *aim13Δ* (BY4741), and *aim37Δ* (BY4741) obtained from Euroscarf (Frankfurt, Germany; Brachmann et al., 1998). The strains Sam37_{ProtA}, Oxa1_{ProtA}, and Fcj1_{ProtA} have been described previously (Kozjak et al., 2003; Frazier et al., 2006; von der Malsburg et al., 2011). A strain expressing Mio27 fused to a C-terminal protein A tag for affinity chromatography was generated by homologous recombination using a cassette consisting of a TEV protease cleavage site, a protein A moiety, and a *HIS3* marker gene (Knop et al., 1999). A similar cassette was transformed into Sam50 Δ ₁₂₀ cells (Kutik et al., 2008) to generate the strain Fcj1_{ProtA} Sam50 Δ ₁₂₀. A fragment encoding a *HIS3* marker gene, a *NOP1* promoter, a protein A moiety, and a TEV protease cleavage site was amplified from genomic DNA derived from Sam37_{ProtA} cells and transformed into Sam50 Δ ₁₂₀ cells to generate the strain Sam37_{ProtA} Sam50 Δ ₁₂₀. A cassette encoding a *kanMX6* module and a *GAL1* promoter was integrated 5' of the *FCJ1* open reading frame by homologous recombination to generate strain Fcj1 \downarrow (YPH499 *fcj1::kanMX6*, *PGAL1-FCJ1*; Longtine et al., 1998).

Growth conditions, isolation of mitochondria, and analysis of protein content

For isolation of mitochondria, cells were grown at 30°C. Typically, cells were cultured in YPG medium (1% [wt/vol] yeast extract, 2% [wt/vol] bacto-peptone, 3% [vol/vol] glycerol). For depletion of Fcj1, the strain Fcj1 \downarrow (YPH499 *fcj1::kanMX6*, *PGAL1-FCJ1*) and the corresponding wild-type strain were precultured in YPGal medium

(1% [wt/vol] yeast extract, 2% [wt/vol] bacto-peptone, 2% [wt/vol] galactose) for 6 h and transferred on YPG medium. After approximately three doubling times, 1% glucose was added to the medium to block expression of the *PGAL1-FCJ1* gene, and cells were harvested after 11 h. Mitochondria were isolated by sequential centrifugation as previously described (Meisinger *et al.*, 2006). Mitochondrial protein content was analyzed by SDS-PAGE and Western blotting. Alternatively, protein complexes were analyzed by solubilization in digitonin buffer (1% [wt/vol] digitonin, 20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% [vol/vol] glycerol, 2 mM phenylmethylsulfonyl fluoride [PMSF]), blue native electrophoresis (Stojanovski *et al.*, 2007b), and Western blotting.

Protein import into isolated mitochondria

In vitro import reactions typically contained 50–80 µg mitochondria (protein amount) diluted in 100 µl import buffer (3% [wt/vol] bovine serum albumin, 250 mM sucrose, 80 mM KCl, 5 mM MgCl₂, 2 mM KH₂PO₄, 5 mM methionine, 10 mM MOPS-KOH, pH 7.2, 4 mM ATP, 4 mM NADH, 5–10 mM creatine phosphate, 100–200 µg/ml creatine kinase; Ryan *et al.*, 2001; Stojanovski *et al.*, 2007b). Radio-labeled precursor proteins generated by in vitro translation in the presence of [³⁵S]methionine (TNT SP6 Quick Coupled kit; Promega, Madison, WI) were added to prewarmed import reactions (20°C/25°C). Samples were transferred on ice after different time points to terminate import reactions. Import of the precursors of ADP/ATP carrier, F₁-ATPase subunit β and cytochrome c₁ was terminated by addition of an AVO mix (8 µM antimycin A, 1 µM valinomycin, 20 µM oligomycin). Where indicated, nonimported precursor proteins were removed by incubation with 50 µg/ml proteinase K on ice for 15 min. Proteinase K was subsequently inactivated by addition of 2 mM PMSF. Mitochondria were washed with SEM buffer (250 mM sucrose, 10 mM MOPS, pH 7.2, 1 mM EDTA) and analyzed by SDS-PAGE or blue native electrophoresis, which was followed by digital autoradiography.

Preparation of yeast whole-cell extracts and affinity chromatography

Yeast cells were cultured in YPG medium at 30°C. Cells were harvested by centrifugation and washed twice with demineralized water and twice with washing buffer (20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% [vol/vol] glycerol; Stroud *et al.*, 2011b; Zerbes *et al.*, 2012). Cells were frozen in liquid nitrogen and ground using a cryomill (20 min, 25 Hz). The resulting whole-cell powder was solubilized in solubilization buffer (20 mM Tris/HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% [vol/vol] glycerol, 1% [wt/vol] digitonin, 2 mM PMSF, 1× EDTA free proteinase inhibitor [Roche, Indianapolis, IN], 30 µg/ml DNase I); this was followed by a clarifying spin. Protein extracts were subsequently applied to IgG affinity chromatography. Unspecifically bound proteins were removed by extensive washing (20 mM Tris/HCl, pH 7.4, 0.5 mM EDTA, 60 mM NaCl, 10% [vol/vol] glycerol, 0.3% [wt/vol] digitonin, 2 mM PMSF). Bound proteins were eluted by TEV protease cleavage, applied to SDS-PAGE, and visualized by Western blotting.

Phospholipid analysis

Isolated mitochondria were subjected to lipid extraction using chloroform/methanol (2:1; vol/vol) as previously described (Folch *et al.*, 1957). The organic phase was subsequently washed with 0.034% MgCl₂ solution (wt/vol), 2 N KCl/methanol (4:1; vol/vol) and methanol/water/chloroform (48:47:3; per vol). For separation of individual phospholipids, two-dimensional TLC using silica gel 60 plates (Merck, Darmstadt, Germany) was applied (first developing solvent:

chloroform/methanol/25% NH₃ [68:35:5; per vol]; second developing solvent: chloroform/acetone/methanol/acetic acid/water [53:20:10:10:5; per vol]). After iodine vapor staining of TLC plates, phospholipids were scraped off and quantified according to Broekhuysse (1968).

Miscellaneous

For assessment of the mitochondrial membrane potential, the potential-sensitive dye dipropylthiadicarbocyanine iodide (DiSC₃(5)) was used (Geissler *et al.*, 2000). For electron microscopy analysis, diamino-benzidine staining and imaging of cells were performed as previously described (von der Malsburg *et al.*, 2011; Zerbes *et al.*, 2012).

ACKNOWLEDGMENTS

We thank Peter Rehling for materials and discussion and Anita Kram and Rinse de Boer for assistance with electron microscopy analysis. This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 746, Excellence Initiative of the German Federal and State Governments (EXC 294 BIOSS; GSC-4 Spemann Graduate School), Bundesministerium für Bildung und Forschung, Landesforschungspreis Baden-Württemberg, and the Austrian Science Fund (project 21429 and DK Molecular Enzymology W901-B05 to G.D.) and was carried out within the research program of the Kluyver Center for Genomics of Industrial Fermentation, which is part of the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research.

REFERENCES

- Alkhaja AK *et al.* (2012). MINOS1 is a conserved component of mitofilin complexes and required for mitochondrial function and cristae organization. *Mol Biol Cell* 23, 247–257.
- Banci L, Bertini I, Cefaro C, Ciofi-Baffoni S, Gallo A, Martinelli M, Sideris DP, Katrakili N, Tokatlidis K (2009). MIA40 is an oxidoreductase that catalyzes oxidative protein folding in mitochondria. *Nat Struct Mol Biol* 16, 198–206.
- Becker T, Böttinger L, Pfanner N (2012). Mitochondrial protein import: from transport pathways to an integrated network. *Trends Biochem Sci* 37, 85–91.
- Becker T, Gebert M, Pfanner N, van der Laan M (2009). Biogenesis of mitochondrial membrane proteins. *Curr Opin Cell Biol* 21, 484–493.
- Becker T, Guiard B, Thornton N, Zufall N, Stroud DA, Wiedemann N, Pfanner N (2010). Assembly of the mitochondrial protein import channel: role of Tom5 in two-stage interaction of Tom40 with the SAM complex. *Mol Biol Cell* 21, 3106–3113.
- Becker T, Pfannschmidt S, Guiard B, Stojanovski D, Milenkovic D, Kutik S, Pfanner N, Meisinger C, Wiedemann N (2008). Biogenesis of the mitochondrial TOM complex: Mim1 promotes insertion and assembly of signal-anchored receptors. *J Biol Chem* 283, 120–127.
- Becker T *et al.* (2011). The mitochondrial import protein Mim1 promotes biogenesis of multispinning outer membrane proteins. *J Cell Biol* 194, 387–395.
- Bien M, Longen S, Wagener N, Chwalla I, Herrmann JM, Riemer J (2010). Mitochondrial disulfide bond formation is driven by intersubunit electron transfer in Erv1 and proofreading by glutathione. *Mol Cell* 37, 516–528.
- Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 14, 115–132.
- Broekhuysse RM (1968). Phospholipids in tissues of the eye. I. Isolation, characterization and quantitative analysis by two-dimensional thin-layer chromatography of diacyl and vinyl-ether phospholipids. *Biochim Biophys Acta* 152, 307–315.
- Chacinska A, Koehler CM, Milenkovic D, Lithgow T, Pfanner N (2009). Importing mitochondrial proteins: machineries and mechanisms. *Cell* 138, 628–644.
- Chacinska A, Pfannschmidt S, Wiedemann N, Kozjak V, Sanjaún Szklarz LK, Schulze-Specking A, Truscott KN, Guiard B, Meisinger C, Pfanner N (2004). Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins. *EMBO J* 23, 3735–3746.

- Chan NC, Lithgow T (2008). The peripheral membrane subunits of the SAM complex function codependently in mitochondrial outer membrane biogenesis. *Mol Biol Cell* 19, 126–136.
- Dabir DV, Leverich EP, Kim SK, Tsai FD, Hirasawa M, Knaff DB, Koehler CM (2007). A role for cytochrome c and cytochrome c peroxidase in electron shuttling from Erv1. *EMBO J* 26, 4801–4811.
- Darshi M, Mendiola VL, Mackey MR, Murphy AN, Koller A, Perkins GA, Ellisman MH, Taylor SS (2011). ChChd3, an inner mitochondrial membrane protein, is essential for maintaining crista integrity and mitochondrial function. *J Biol Chem* 286, 2918–2932.
- Dimmer KS, Papić D, Schumann B, Sperl D, Krümpe K, Walther DM, Rapaport D (2012). A crucial role of Mim2 in the biogenesis of mitochondrial outer membrane proteins. *J Cell Sci* 125, 3464–3473.
- Dolezal P, Likic V, Tachezy J, Lithgow T (2006). Evolution of the molecular machines for protein import into mitochondria. *Science* 313, 314–318.
- Dukanovic J, Dimmer KS, Bonnefoy N, Krümpe K, Rapaport D (2009). Genetic and functional interactions between the mitochondrial outer membrane proteins Tom6 and Sam37. *Mol Cell Biol* 29, 5975–5988.
- Dukanovic J, Rapaport D (2011). Multiple pathways in the integration of proteins into the mitochondrial outer membrane. *Biochim Biophys Acta* 1808, 971–980.
- Endo T, Yamano K (2009). Multiple pathways for mitochondrial protein traffic. *Biol Chem* 390, 723–730.
- Endo T, Yamano K, Kawano S (2011). Structural insight into the mitochondrial protein import system. *Biochim Biophys Acta* 1808, 955–970.
- Folch J, Lees M, Stanley GHS (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J Biol Chem* 226, 497–509.
- Frazier AE, Taylor RD, Mick DU, Warscheid B, Stoepel N, Meyer HE, Ryan MT, Guiard B, Rehling P (2006). Mdm38 interacts with ribosomes and is a component of the mitochondrial protein export machinery. *J Cell Biol* 172, 553–564.
- Frey TG, Mannella CA (2000). The internal structure of mitochondria. *Trends Biochem Sci* 25, 319–324.
- Gebert N *et al.* (2009). Mitochondrial cardiolipin involved in outer-membrane protein biogenesis: implications for Barth syndrome. *Curr Biol* 19, 2133–2139.
- Geissler A, Krimmer T, Bömer U, Guiard B, Rassow J, Pfanner N (2000). Membrane potential-driven protein import into mitochondria: the sorting sequence of cytochrome *b₂* modulates the $\Delta\psi$ -dependence of translocation of the matrix-targeting sequence. *Mol Biol Cell* 11, 3977–3991.
- Gentile I, Gabriel K, Beech P, Waller R, Lithgow T (2004). The Omp85 family of proteins is essential for outer membrane biogenesis in mitochondria and bacteria. *J Cell Biol* 164, 19–24.
- Grumbt B, Stroobant V, Terziyska N, Israel L, Hell K (2007). Functional characterization of Mia40p, the central component of the disulfide relay system of the mitochondrial intermembrane space. *J Biol Chem* 282, 37461–37470.
- Habib SJ, Waizenegger T, Lech M, Neupert W, Rapaport D (2005). Assembly of the TOB complex of mitochondria. *J Biol Chem* 280, 6434–6440.
- Habib SJ, Waizenegger T, Niewianda A, Paschen SA, Neupert W, Rapaport D (2007). The N-terminal domain of Tob55 has a receptor-like function in the biogenesis of mitochondrial β -barrel proteins. *J Cell Biol* 176, 77–88.
- Harner M, Körner C, Walther D, Mokranjac D, Kaesmacher J, Welsch U, Griffith J, Mann M, Reggiori F, Neupert W (2011). The mitochondrial contact site complex, a determinant of mitochondrial architecture. *EMBO J* 30, 4356–4370.
- Head BP, Zulaika M, Ryazantsev S, van der Bliek AM (2011). A novel mitochondrial outer membrane protein, MOMA-1, that affects cristae morphology in *Caenorhabditis elegans*. *Mol Biol Cell* 22, 831–841.
- Herrmann JM (2011). MINOS is plus: a mitofilin complex for mitochondrial membrane contacts. *Dev Cell* 21, 599–600.
- Hoppins S, Collins SR, Cassidy-Stone A, Hummel E, DeVay RM, Lackner LL, Westermann B, Schuldiner M, Weissman JS, Nunnari J (2011). A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria. *J Cell Biol* 195, 323–340.
- Hoppins SC, Nargang FE (2004). The Tim8-Tim13 complex of *Neurospora crassa* functions in the assembly of proteins into both mitochondrial membranes. *J Biol Chem* 279, 12396–12405.
- Hulett JM, Lueder F, Chan NC, Perry AJ, Wolyniec P, Likic VA, Gooley PR, Lithgow T (2008). The transmembrane segment of Tom20 is recognized by Mim1 for docking to the mitochondrial TOM complex. *J Mol Biol* 376, 694–704.
- Ishikawa D, Yamamoto H, Tamura Y, Moritoh K, Endo T (2004). Two novel proteins in the mitochondrial outer membrane mediate β -barrel protein assembly. *J Cell Biol* 166, 621–627.
- John GB, Shang Y, Li L, Renken C, Mannella CA, Selker JML, Rangell L, Bennett MJ, Zha J (2005). The mitochondrial inner membrane protein mitofilin controls cristae morphology. *Mol Biol Cell* 16, 1543–1554.
- Kawano S, Yamano K, Naoé M, Momose T, Terao K, Nishikawa S, Watanabe N, Endo T (2009). Structural basis of yeast Tim40/Mia40 as an oxidative translocator in the mitochondrial intermembrane space. *Proc Natl Acad Sci USA* 106, 14403–14407.
- Keil P, Pfanner N (1993). Integration of MOM22 into the mitochondrial outer membrane strictly depends on surface receptors. *FEBS Lett* 321, 197–200.
- Kemper C, Habib SJ, Engl G, Heckmeyer P, Dimmer KS, Rapaport D (2008). Integration of tail-anchored proteins into the mitochondrial outer membrane does not require any known import components. *J Cell Sci* 121, 1990–1998.
- Knop M, Siegers K, Pereira G, Zachariae W, Winsor B, Nasmyth K, Schieble E (1999). Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast* 15, 963–972.
- Knowles TJ, Jeeves M, Bobat S, Dancea F, McClelland D, Palmer T, Overduin M, Henderson IR (2008). Fold and function of polypeptide transport-associated domains responsible for delivering unfolded proteins to membranes. *Mol Microbiol* 68, 1216–1227.
- Koehler CM, Tiensohn HL (2009). Redox regulation of protein folding in the mitochondrial intermembrane space. *Biochim Biophys Acta* 1793, 139–145.
- Körner C, Barrera M, Dukanovic J, Eyd K, Harner M, Rabl R, Vogel F, Rapaport D, Neupert W, Reichert AS (2012). The C-terminal domain of Fcj1 is required for formation of crista junctions and interacts with the TOB/SAM complex in mitochondria. *Mol Biol Cell* 23, 2143–2155.
- Kozjak V, Wiedemann N, Milenkovic D, Lohaus C, Meyer HE, Guiard B, Meisinger C, Pfanner N (2003). An essential role of Sam50 in the protein sorting and assembly machinery of the mitochondrial outer membrane. *J Biol Chem* 278, 48520–48523.
- Kutik S *et al.* (2008). Dissecting membrane insertion of mitochondrial β -barrel proteins. *Cell* 132, 1011–1024.
- Longtine MS, McKenzie A, III, Demarini DJ, Shah NG, Wach A, Brachet A, Philippsen P, Pringle JR (1998). Additional modules for versatile and economical PCR-based gene deletion and modification in *S. cerevisiae*. *Yeast* 14, 953–961.
- Mannella CA (2006). The relevance of mitochondrial membrane topology to mitochondrial function. *Biochim Biophys Acta* 1762, 140–147.
- Meisinger C, Pfanner N, Truscott KN (2006). Isolation of yeast mitochondria. *Methods Mol Biol* 313, 33–39.
- Meisinger C, Ryan MT, Hill K, Model K, Lim JH, Sickmann A, Müller H, Meyer HE, Wagner R, Pfanner N (2001). Protein import channel of the outer mitochondrial membrane: a highly stable Tom40-Tom22 core structure differentially interacts with preproteins, small Tom proteins, and import receptors. *Mol Cell Biol* 21, 2337–2348.
- Meisinger C *et al.* (2004). The mitochondrial morphology protein Mdm10 functions in assembly of the preprotein translocase of the outer membrane. *Dev Cell* 7, 61–71.
- Mesecke N, Terziyska N, Kozany C, Baumann F, Neupert W, Hell K, Herrmann JM (2005). A disulfide relay system in the intermembrane space of mitochondria that mediates protein import. *Cell* 121, 1059–1069.
- Mihara K (2003). Cell biology: moving inside membranes. *Nature* 424, 505–506.
- Milenkovic D, Kozjak V, Wiedemann N, Lohaus C, Meyer HE, Guiard B, Pfanner N, Meisinger C (2004). Sam35 of the mitochondrial protein sorting and assembly machinery is a peripheral outer membrane protein essential for cell viability. *J Biol Chem* 279, 22781–22785.
- Model K, Meisinger C, Prinz T, Wiedemann N, Truscott KN, Pfanner N, Ryan MT (2001). Multistep assembly of the protein import channel of the mitochondrial outer membrane. *Nat Struct Biol* 8, 361–370.
- Mun JY, Lee THL, Kim JH, Yoo BH, Bahk YY, Koo H-S, Han SS (2010). *Caenorhabditis elegans* mitofilin homologs control the morphology of mitochondrial cristae and influence reproduction and physiology. *J Cell Physiol* 224, 748–756.
- Neupert W, Herrmann JM (2007). Translocation of proteins into mitochondria. *Annu Rev Biochem* 76, 723–749.
- Ott C *et al.* (2012). Sam50 functions in mitochondrial intermembrane space bridging and biogenesis of respiratory complexes. *Mol Cell Biol* 32, 1173–1188.

- Papić D, Krumpe K, Dukanovic J, Dimmer KS, Rapaport D (2011). Multispan mitochondrial outer membrane protein Ugo1 follows a unique Mim1-dependent import pathway. *J Cell Biol* 194, 397–405.
- Paschen SA, Neupert W, Rapaport D (2005). Biogenesis of β -barrel membrane proteins of mitochondria. *Trends Biochem Sci* 30, 575–582.
- Paschen SA, Waizenegger T, Stan T, Preuss M, Cyrklaff M, Hell K, Rapaport D, Neupert W (2003). Evolutionary conservation of biogenesis of β -barrel membrane proteins. *Nature* 426, 862–866.
- Popov-Čeleketić J, Waizenegger T, Rapaport D (2008). Mim1 functions in an oligomeric form to facilitate the integration of Tom20 into the mitochondrial outer membrane. *J Mol Biol* 376, 671–680.
- Rabl R *et al.* (2009). Formation of cristae and crista junctions in mitochondria depends on antagonism between Fc1 and Su *e/g*. *J Cell Biol* 185, 1047–1063.
- Rao S, Schmidt O, Harbauer AB, Schönfisch B, Guiard B, Pfanner N, Meisinger C (2012). Biogenesis of the preprotein translocase of the outer mitochondrial membrane: protein kinase A phosphorylates the precursor of Tom40 and impairs its import. *Mol Biol Cell* 23, 1618–1627.
- Ryan MT, Voos W, Pfanner N (2001). Assaying protein import into mitochondria. *Methods Cell Biol* 65, 189–215.
- Sánchez-Pulido L, Devos D, Genevrois S, Vicente M, Valencia A (2003). POTRA: a conserved domain in the FtsQ family and a class of β -barrel outer membrane proteins. *Trends Biochem Sci* 28, 523–526.
- Sikorski RS, Hieter P (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122, 19–27.
- Stojanovski D, Guiard B, Kozjak-Pavlovic V, Pfanner N, Meisinger C (2007a). Alternative function for the mitochondrial SAM complex in biogenesis of α -helical TOM proteins. *J Cell Biol* 179, 881–893.
- Stojanovski D, Milenkovic D, Müller JM, Gabriel K, Schulze-Specking A, Baker MJ, Ryan MT, Guiard B, Pfanner N, Chacinska A (2008). Mitochondrial protein import: precursor oxidation in a ternary complex with disulfide carrier and sulfhydryl oxidase. *J Cell Biol* 183, 195–202.
- Stojanovski D, Pfanner N, Wiedemann N (2007b). Import of proteins into mitochondria. *Methods Cell Biol* 80, 783–806.
- Stroud DA *et al.* (2011a). Biogenesis of mitochondrial β -barrel proteins: the POTRA domain is involved in precursor release from the SAM complex. *Mol Biol Cell* 22, 2823–2833.
- Stroud DA, Oeljeklaus S, Wiese S, Bohnert M, Lewandrowski U, Sickmann A, Guiard B, van der Laan M, Warscheid B, Wiedemann N (2011b). Composition and topology of the endoplasmic reticulum-mitochondria encounter structure. *J Mol Biol* 413, 743–750.
- Thornton N, Stroud DA, Milenkovic D, Guiard B, Pfanner N, Becker T (2010). Two modular forms of the mitochondrial sorting and assembly machinery are involved in biogenesis of α -helical outer membrane proteins. *J Mol Biol* 396, 540–549.
- van der Laan M, Bohnert M, Wiedemann N, Pfanner N (2012). Role of MINOS in mitochondrial membrane architecture and biogenesis. *Trends Cell Biol* 22, 185–192.
- von der Malsburg K *et al.* (2011). Dual role of mitofilin in mitochondrial membrane organization and protein biogenesis. *Dev Cell* 21, 694–707.
- Waizenegger T, Habib SJ, Lech M, Mokranjac D, Paschen SA, Hell K, Neupert W, Rapaport D (2004). Tob38, a novel essential component in the biogenesis of β -barrel proteins of mitochondria. *EMBO Rep* 5, 704–709.
- Wiedemann N, Kozjak V, Chacinska A, Schönfisch B, Rospert S, Ryan MT, Pfanner N, Meisinger C (2003). Machinery for protein sorting and assembly in the mitochondrial outer membrane. *Nature* 424, 565–571.
- Wiedemann N, Truscott KN, Pfannschmidt S, Guiard B, Meisinger C, Pfanner N (2004). Biogenesis of the protein import channel Tom40 of the mitochondrial outer membrane: intermembrane space components are involved in an early stage of the assembly pathway. *J Biol Chem* 279, 18188–18194.
- Xie J, Marusich MF, Souda P, Whitelegge J, Capaldi RA (2007). The mitochondrial inner membrane protein mitofilin exists as a complex with SAM50, metaxins 1 and 2, coiled-coil-helix coiled-coil-helix domain-containing protein 3 and 6 and DnaJC11. *FEBS Lett* 581, 3545–3549.
- Zerbes RM *et al.* (2012). Role of MINOS in mitochondrial membrane architecture: cristae morphology and outer membrane interactions differentially depend on mitofilin domains. *J Mol Biol* 422, 183–191.
- Zick M, Rabl R, Reichert AS (2009). Cristae formation—linking ultrastructure and function of mitochondria. *Biochim Biophys Acta* 1793, 5–19.