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

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.

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 Tienson, 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; Gentle et al., 2004; Hoppins and Nargang, 2004; Habib et al., 2005; Chan and Lighthow, 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-Celeketic et al., 2008; Thornton et al., 2010; Dukanovic and Rapaport, 2011; Papic 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 pull-down 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 immunoblotting. Extracts of the yeast cells were prepared using the nonionic detergent digitonin and subjected to immunoblotting. Bound proteins were eluted by cleavage with TEV protease. Fcj1ProtA and Mio27ProtA 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, Fcj1ProtA 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, Mio27ProtA 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 (Fcj1ProtA and Mio27ProtA) were subjected to IgG affinity chromatography, elution with TEV protease, and analysis by SDSPAGE and immunoblotting. Load, 1.5%; elution, 100%. Mio27, TEV-cleaved form of Mio27ProtA; 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 (Sam50Δ120; 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.

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

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...
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 Δψ was comparable with that of wild-type mitochondria (Figure 5A). For fcj1Δ mitochondria, it has been reported that Δψ is partially decreased, and the Δψ-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 c1 and ADP/ATP carrier with an efficiency close to that of wild-type mitochondria (Figure 5B and C), indicating that mitofilin/FCJ1 is not required for β-barrel assembly.

**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 assembly intermediate I, which represents interaction of the precursor with the TOM 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 and 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 Δψ was comparable with that of wild-type mitochondria (Figure 5A). For fcj1Δ mitochondria, it has been reported that Δψ is partially decreased, and the Δψ-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 c1 and ADP/ATP carrier with an efficiency close to that of wild-type mitochondria (Figure 5B and C), indicating that mitofilin/FCJ1 is 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↓YPH499 (Δfcj1::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 DSC6(5). (B) The [35S]labeled precursors of F$_{1}$-ATPase subunit β (Fj_{β}) and cytochrome c$_{1}$ (Cyt. c$_{1}$) 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.

fcj1Δ 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 Sam50Δ,20 mitochonrdia with Tom40 assembly in fcj1Δ 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 fcj1Δ 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 Sam37ProtA 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 fcj1Δ 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 fcj1Δ 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 (Harnre 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; Harnre 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
Mitofilin and outer membrane biogenesis

We dissected the biogenesis pathway of Tom40 and FCJ1. Moreover, we observed that the phospholipid profiles of β-burg membrane (Harner et al., 2011; Alkhaja et al., 2012), it can be excluded 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 aim3Δ 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 aim3Δ (BY4741) obtained from Euroscarf (Frankfurt, Germany; Brachmann et al., 1998). The strains Sam37ProtA, Oxa1ProtA, and Fcj1ProtA 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 module and a 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).

FIGURE 6: Mitofilin/FCJ1 is required at an early stage of Tom40 biogenesis. (A) [35S]-labeled Tom40 was imported into Sam37ProtA and Sam37ProtA Sam50A Δmio10 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 ± 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).

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 Fcj1Δ (YPH499 fcj1Δ::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 PGA1-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/Cl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% [vol/vol] glycerol, 2 mM phe- nylmethylsulfonyl 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 Na2HPO4, 5–10 mM creatine phosphate, 100–200 μg/ml creatine kinase; Ryan et al., 2001; Stojanovski et al., 2007b). Radio-labeled precursor proteins were generated by in vitro translation in the presence of [35S]methionine (TNT SP6 Quick Coupled kit; Promega, Madison, WI) and 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/Cl, 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/Cl, 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 clarification step. Protein extracts were subsequently applied to IgG affinity chromatography. Unspecifically bound proteins were removed by extensive washing (20 mM Tris/Cl, 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/25% NH4Cl [68:35:5; per vol]; second developing solvent: chloroform/aceton/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 Broekhuysen (1968).

**Miscellaneous**

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

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