

Sam37 is crucial for formation of the mitochondrial TOM–SAM supercomplex, thereby promoting β -barrel biogenesis

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Biogenesis of mitochondrial β -barrel proteins requires two preprotein translocases, the general translocase of the outer membrane (TOM) and the sorting and assembly machinery (SAM). TOM and SAM form a supercomplex that promotes transfer of β -barrel precursors. The SAM core complex contains the channel protein Sam50, which cooperates with Sam35 in precursor recognition, and the peripheral membrane protein Sam37. The molecular function of Sam37 has been unknown. We report that Sam37 is crucial for formation of the TOM–SAM supercomplex. Sam37 interacts with the receptor domain of Tom22 on the cytosolic side of the mitochondrial outer membrane and links TOM and SAM complexes. Sam37 thus promotes efficient transfer of β -barrel precursors to the SAM complex. We conclude that Sam37 functions as a coupling factor of the translocase supercomplex of the mitochondrial outer membrane.

Introduction

The mitochondrial outer membrane contains two membrane-integrated preprotein translocases promoting β -barrel biogenesis. The translocase of the outer membrane (TOM) is responsible for importing the vast majority of nuclear-encoded mitochondrial proteins (Koehler, 2004; Dolezal et al., 2006; Neupert and Herrmann, 2007; Chacinska et al., 2009; Endo and Yamano, 2010; Schleiff and Becker, 2011). The sorting and assembly machinery (SAM), also termed topogenesis of mitochondrial outer membrane β -barrel proteins (TOB), is dedicated to membrane integration and assembly of β -barrel proteins of the outer membrane (Endo and Yamano, 2010; Dukanovic and Rapaport, 2011; Hewitt et al., 2011; Höhr et al., 2015). Upon synthesis on cytosolic ribosomes, the precursors of mitochondrial β -barrel proteins are recognized by TOM receptors and imported by the Tom40 channel to the intermembrane space side of the outer membrane (Krimmer et al., 2001; Paschen et al., 2003; Wiedemann et al., 2003; Humphries et al., 2005). Small translocase of inner membrane chaperones bind to the β -barrel precursors and prevent their aggregation (Hoppins and Nargang, 2004; Wiedemann et al., 2004). The precursor proteins are inserted into the outer membrane by the SAM complex (Ishikawa et al., 2004; Qiu et al., 2013).

The SAM complex contains three main subunits (Yamano et al., 2010; Klein et al., 2012; Qiu et al., 2013). Sam50 (Tob55), which has been conserved from bacteria (BamA) to humans, forms a membrane-integrated β -barrel channel (Kozjak et al., 2003; Paschen et al., 2003; Gentle et al., 2004; Humphries et al., 2005; Bredemeier et al., 2007; Kozjak-Pavlovic et al., 2007; Kutik et al., 2008; Walther et al., 2009, 2010; Ulrich et al., 2012). Sam35 and Sam37 are peripheral membrane proteins exposed on the cytosolic side of the mitochondrial outer membrane (Gratzer et al., 1995; Ryan et al., 1999; Wiedemann et al., 2003; Ishikawa et al., 2004; Milenkovic et al., 2004; Waizenegger et al., 2004; Lackey et al., 2011). Sam35 cooperates with Sam50 in the recognition of β -barrel precursors (Chan and Lithgow, 2008; Kutik et al., 2008). Though Sam37 has been identified as the first subunit of the SAM complex (Wiedemann et al., 2003), its molecular function has not been elucidated so far. The current findings suggest that Sam37 may function as an assembly factor that promotes maturation of β -barrel precursors (Chan and Lithgow, 2008; Dukanovic et al., 2009; Becker et al., 2010; Lackey et al., 2011).

The TOM and SAM complexes directly interact with each other, forming a TOM–SAM supercomplex that facilitates the transfer of β -barrel precursors (Qiu et al., 2013). The central receptor, Tom22, was found to function as the binding partner at the TOM side. Though the findings suggested that Tom22

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Abbreviations used in this paper: BMOE, bismaleimidoethane; DSG, disuccinimidyl glutarate; Ni-NTA, nickel–nitrilotriacetic acid; SAM, sorting and assembly machinery; TOM, translocase of the outer membrane; Tom22_{CD}, cytosolic domain of Tom22.

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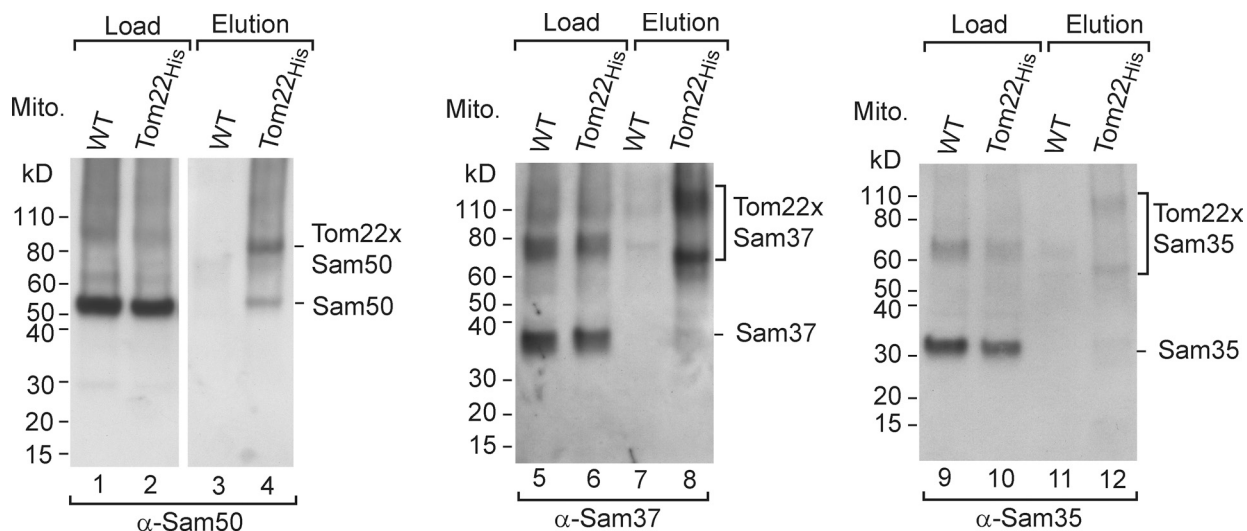


Figure 1. **Sam37 is the major cross-linking partner of Tom22 at the SAM complex.** Wild-type (WT) and Tom22^{His} yeast mitochondria were treated with the cross-linking reagent DSG. Subsequently, mitochondria were lysed under denaturing conditions and subjected to Ni-NTA chromatography. Proteins were analyzed by SDS-PAGE and immunodetection with the indicated antisera. Load, 0.5%; elution, 100%.

directly interacts with the SAM complex, the binding partner at the SAM side has not been identified.

We report that Sam37 binds to Tom22 and is required for the formation of the TOM–SAM supercomplex. Sam37 is crucial for coupling TOM and SAM complexes and thus promotes β -barrel transfer between the protein translocases of the outer membrane.

Results and discussion

Early role of Sam37 in β -barrel biogenesis

To define the SAM subunits that are in close proximity to Tom22 in the native membrane environment, we performed chemical cross-linking with intact yeast mitochondria using a homobifunctional amino-reactive cross-linking reagent. The mitochondria were derived from a yeast strain expressing Tom22 with a His tag to allow isolation of Tom22-containing cross-linking products by affinity purification (Qiu et al., 2013). Tom22–SAM cross-linking products were detected by immunodecoration. In addition to a cross-linking product between Tom22 and Sam50 (Fig. 1, lane 4; Qiu et al., 2013), we observed the efficient formation of Tom22–Sam37 cross-linking products (Fig. 1, lane 8). Tom22–Sam35 cross-linking products were only found with low yield (Fig. 1, lane 12). Thus, cross-linking of Tom22 to Sam37 was considerably more efficient than that to Sam50 or Sam35. These results indicate that Sam37 is in close proximity to Tom22 in intact mitochondria, raising the possibility that Sam37 may be involved in TOM–SAM coupling.

Because the cross-linking assay only indicates spatial proximity, but not the function of Sam37, we sought functional approaches to address a putative early role of Sam37 in the transfer of β -barrel precursors to the SAM complex. So far, Sam37 has been reported to be involved in the assembly steps that promote precursor maturation and eventual release from the SAM complex (Chan and Lithgow, 2008; Dukanovic et al., 2009; Becker et al., 2010; Lackey et al., 2011). An early role of Sam37 has been difficult to study because upon deletion of *SAM37*, the levels of Sam35, Sam50, Tom22, and Tom40 were strongly reduced when

yeast cells were grown at the usual temperatures of 25–30°C (Stojanovski et al., 2007; Chan and Lithgow, 2008; Dukanovic et al., 2009). Therefore, indirect effects of the lack of Sam37 could not be excluded. We established several approaches to minimize indirect effects of a deletion of *SAM37* and to study the role of Sam37 directly. (a) Optimization of the growth conditions of *sam37* Δ yeast cells: We screened for conditions that diminished the decrease of the mitochondrial levels of SAM and TOM subunits in *sam37* Δ cells. We obtained the best result upon cell growth at very low temperature (19°C), leading to a moderate reduction of the levels of translocase subunits (Fig. S1 A). (b) In vitro complementation of *sam37* Δ mitochondria by importing wild-type Sam37: The precursor of Sam37 was synthesized in large amounts in a cell-free translation system based on wheat germ extract (Becker et al., 2010). Sam37 was imported into purified *sam37* Δ mitochondria, followed by import of β -barrel precursors to determine whether SAM functions were restored by imported Sam37. Thus, Sam37-specific functions could be studied in organello. (c) Overexpression of Sam35 in *sam37* Δ yeast to restore the mitochondrial levels of Sam35 (see Results and discussion section Overexpression of Sam35 or Tom6 . . .).

To determine whether Sam37 was required at an early stage of β -barrel biogenesis, distinct import and assembly steps of the Tom40 precursor were studied by blue native electrophoresis of mitochondria lysed with the mild detergent digitonin. Upon import of ³⁵S-labeled Tom40 precursor into isolated mitochondria, three stages can be separated: intermediate I representing the binding of the precursor to SAM, intermediate II that is released from SAM, and assembly into the mature TOM complex (Fig. 2 A, lanes 1 and 2; Paschen et al., 2003; Wiedemann et al., 2003; Chan and Lithgow, 2008; Dukanovic et al., 2009; Becker et al., 2010). We used mitochondria isolated from yeast grown at 19°C and observed a strong reduction of intermediate I (SAM intermediate), as well as of the subsequent assembly steps, in *sam37* Δ mitochondria (Fig. 2 A, lanes 3 and 4). When in vitro-synthesized Sam37 was first imported into *sam37* Δ mitochondria, the subsequent import of [³⁵S]Tom40 was restored (Fig. 2 A, lanes 5 and 6), suggesting Sam37-specific formation of intermediate I (SAM-bound Tom40).

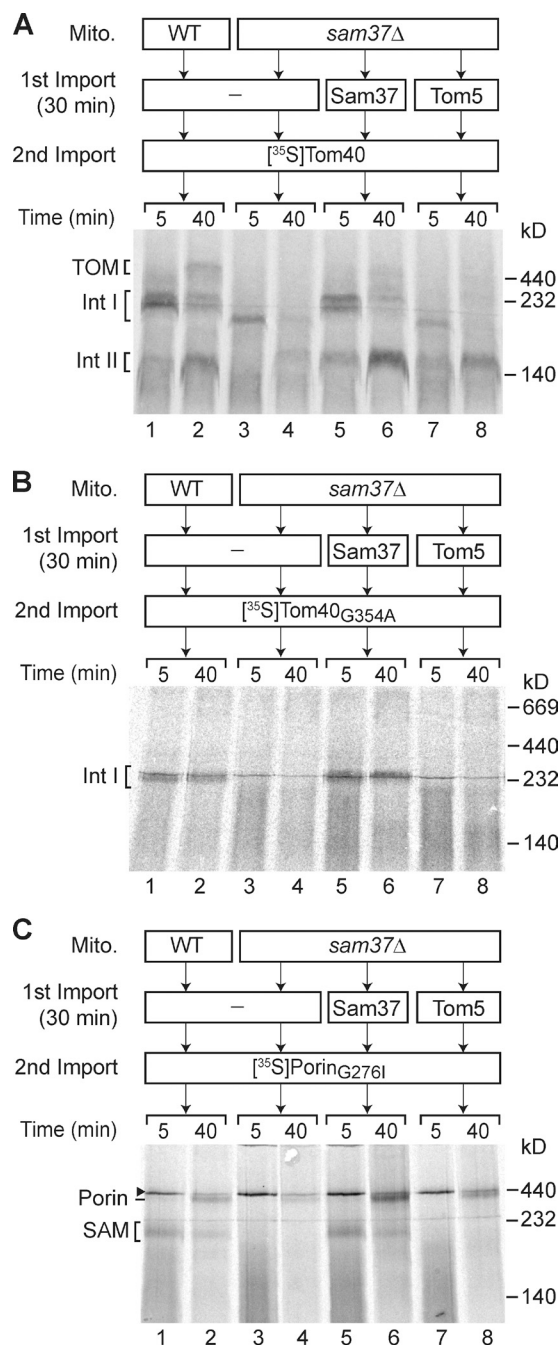


Figure 2. Sam37 is required for binding of β -barrel proteins to the SAM complex. ^{35}S -labeled precursors of Tom40 (A), Tom40_{G354A} (B), or Porin_{G276I} (C) were imported into wild-type (WT) or $sam37\Delta$ yeast mitochondria (cells were grown at 19°C). Where indicated, recombinant amounts of Sam37 or Tom5 were imported into the mitochondria before the incubation with ^{35}S -labeled precursor proteins. The mitochondria were lysed with digitonin and analyzed by blue native electrophoresis and autoradiography. Int I, intermediate I; Int II, intermediate II of Tom40 assembly. The arrowhead indicates an unspecific band.

To directly address whether Sam37 restored the binding of the Tom40 precursor to SAM, we imported a Tom40 variant (Gly354 replaced by Ala), which is not released from the SAM complex and thus can be selectively used to study the precursor–SAM interaction (Kutik et al., 2008). The binding of Tom40_{G354A} to SAM was strongly inhibited in the absence of Sam37 but restored upon in vitro complementation of the mu-

tant mitochondria with Sam37 (Fig. 2 B, lanes 3–6). We asked whether Sam37 also promoted binding of other β -barrel precursors such as Porin to the SAM complex. The assembly of Porin is impaired in the absence of Sam37 (Fig. S1 B), yet the wild-type Porin precursor does not form a blue native stable SAM intermediate (Wiedemann et al., 2003; Dukanovic et al., 2009). We used a Porin variant (Gly276 replaced by Ile), which is imported more slowly and thus can be observed in association with SAM (Kutik et al., 2008). Formation of the SAM-bound stage of Porin_{G276I} was inhibited in $sam37\Delta$ mitochondria and restored by import of Sam37 (Fig. 2 C, lanes 3 and 5). As a control, we show that in vitro–imported Tom5 did not restore the binding of β -barrel precursors to SAM in $sam37\Delta$ mitochondria (Fig. 2, A–C, lanes 7 and 8).

We conclude that Sam37 is in close proximity to Tom22 and plays an early role in the biogenesis of β -barrel proteins. Sam37 is required for an efficient binding of β -barrel precursors to the SAM complex.

Sam37 is required for TOM–SAM supercomplex formation

To determine whether Sam37 is required for the formation of the TOM–SAM supercomplex, we used mitochondria with His-tagged Tom22 and performed an affinity purification under nondenaturing conditions upon lysis of mitochondria with digitonin. TOM and SAM subunits were coeluted, demonstrating copurification of the TOM–SAM supercomplex (Fig. 3 A, lane 5; Qiu et al., 2013). When SAM37 was deleted, however, the copurification of SAM subunits with Tom22_{His} was virtually blocked, whereas the other TOM subunits were efficiently copurified (Fig. 3 A, lane 6). We conclude that a lack of Sam37 inhibits copurification of the TOM–SAM supercomplex.

To study the role of Sam37 for the TOM–SAM supercomplex in the native membrane environment, we used cross-linking of Tom22 to Sam50 as an assay (Fig. 1; Qiu et al., 2013). In mitochondria lacking Sam37, the cross-linking product between Sam50 and Tom22_{His} was hardly detectable (Fig. 3 B, lane 6), indicating that Sam37 is crucial for linking TOM and SAM complexes.

We used a fusion protein between a portion of cytochrome b_2 and Tom40 (Shiota et al., 2012; Qiu et al., 2013) to functionally address a role of Sam37 in the TOM–SAM supercomplex. Upon import into mitochondria in the absence of an inner membrane potential, the b_2 –Tom40 fusion protein accumulates in the TOM–SAM supercomplex, forming a TOM–SAM–preprotein complex of ~700 kD (Fig. 3 C, lane 1); the fusion protein spans both TOM and SAM simultaneously (Qiu et al., 2013). Accumulation of b_2 –Tom40 in the TOM–SAM supercomplex was strongly inhibited in $sam37\Delta$ mitochondria (Fig. 3 C, lane 2). Import of in vitro–synthesized Sam37 into $sam37\Delta$ mitochondria promoted formation of the TOM–SAM–preprotein complex (Fig. 3 C, lane 3).

Collectively, our findings indicate that Sam37 is required for formation of the TOM–SAM supercomplex and thus promotes the efficient transfer of β -barrel precursors from TOM to SAM.

Overexpression of Sam35 or Tom6 does not restore formation of the TOM–SAM supercomplex in the absence of Sam37

Overexpression of Sam35 or Tom6 was shown to stimulate the growth of Sam37-deficient yeast (Meisinger et al., 2007; Chan and Lithgow, 2008; Dukanovic et al., 2009; Becker et al., 2010).

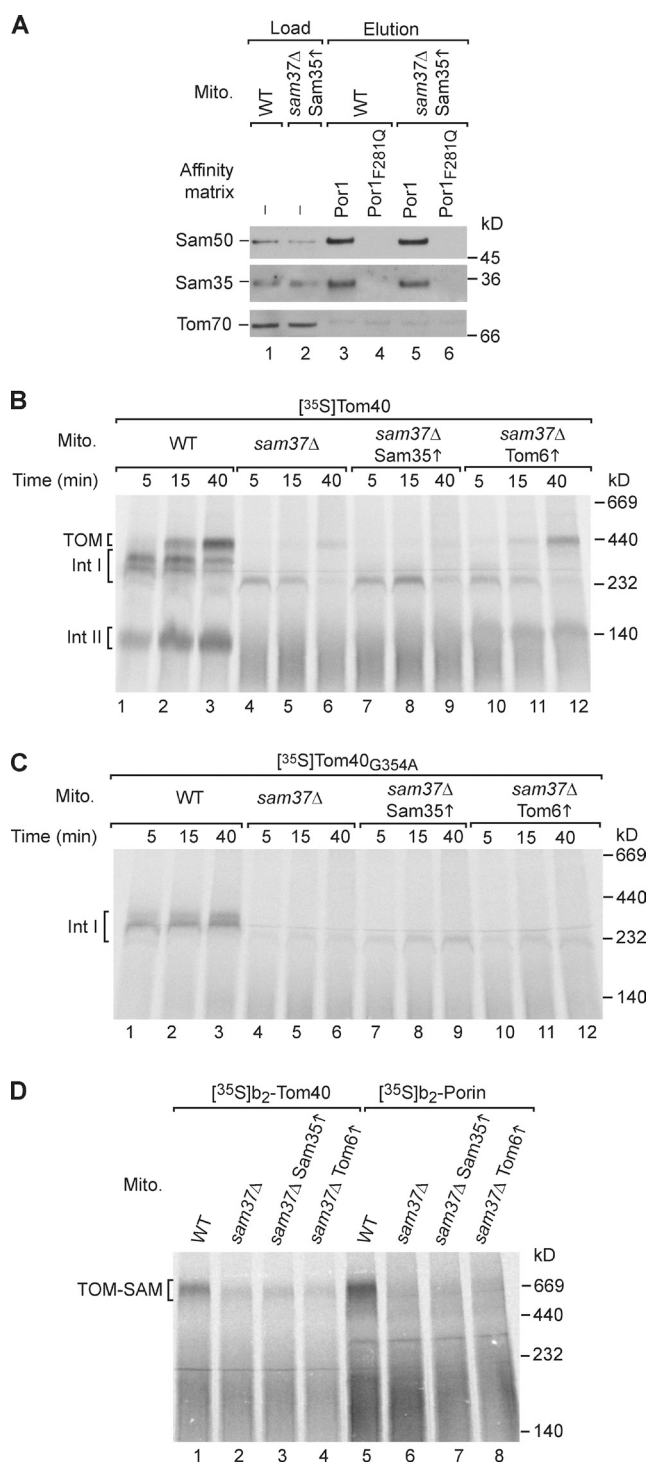


Figure 4. Overexpression of Sam35 or Tom6 does not compensate for the early role of Sam37 in β -barrel biogenesis. (A) Wild-type (WT) and *sam37Δ* Sam35[↑] mitochondria were lysed with digitonin and incubated with the recombinant β -signal of Porin (Por1) or the mutant version (Por1^{F281Q}) coupled to an affinity matrix. Load and elution fractions were analyzed by SDS-PAGE and immunodetection. Load, 40%; elution, 100%. (B and C) [³⁵S]-labeled precursors of Tom40 (B) or Tom40^{G354A} (C) were imported into wild-type, *sam37Δ*, *sam37Δ* Sam35[↑], and *sam37Δ* tom6[↑] mitochondria. The mitochondria were lysed with digitonin and analyzed by blue native electrophoresis and autoradiography. Int I, intermediate I; Int II, intermediate II of Tom40 assembly. (D) [³⁵S]-labeled b₂-Tom40 and b₂-Porin were imported into wild-type, *sam37Δ*, *sam37Δ* Sam35[↑], and *sam37Δ* tom6[↑] mitochondria after dissipation of the membrane potential. Mitochondria were lysed in digitonin, and samples were analyzed by blue native electrophoresis and autoradiography.

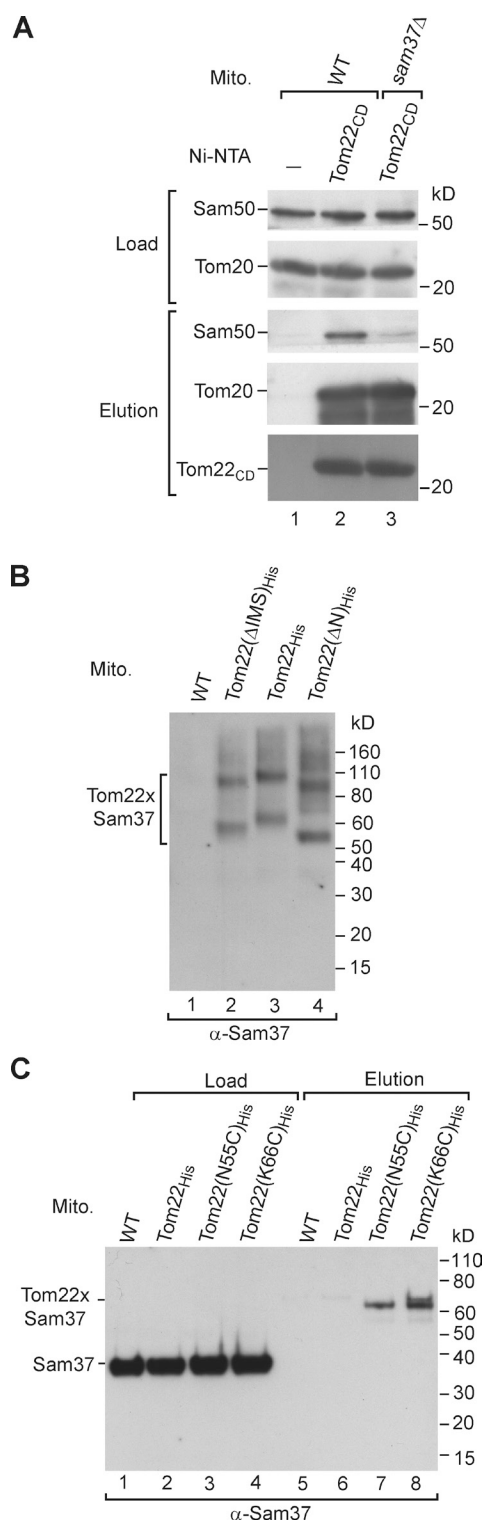


Figure 5. Sam37 interacts with Tom22^{CD}. (A) Wild-type (WT) and *sam37Δ* mitochondria were lysed with digitonin and incubated with an affinity matrix coated with Tom22^{CD} (cells were grown at 19°C). Load and elution fractions were analyzed by SDS-PAGE and immunodetection with the indicated antisera (top four panels) or Coomassie staining (bottom). Load, 2%; elution, 100%. (B) Wild-type, Tom22^{CD}, Tom22^{CD} His, and Tom22^{CD} His mitochondria were treated with the cross-linking reagent DSG and subjected to Ni-NTA chromatography as described in the legend of Fig. 1. Elution, 100%. (C) Wild-type, Tom22^{CD}, Tom22^{CD} His, and Tom22^{CD} His mitochondria were treated with the cross-linking reagent BMOE and subjected to Ni-NTA chromatography as described in the legend of Fig. 1. Load, 0.5%; elution, 100%.

of 8 Å. Both cysteine residues were cross-linked to Sam37, with Cys66 yielding a higher efficiency, whereas wild-type Tom22 did not yield a cross-linking product, confirming the specificity of the approach (Fig. 5 C, lanes 6–8).

We conclude that Sam37 interacts with the cytosolic receptor domain of Tom22, in particular with the second half of the receptor domain. The Tom22–Sam37 interaction thus provides an explanation of why Sam37 is exposed on the cytosolic side of the outer membrane.

Conclusions

We report that the peripheral SAM subunit Sam37 plays an important role in the transfer of β -barrel precursor proteins from the TOM complex to the SAM complex. Sam37 interacts with the receptor domain of Tom22. The Tom22–Sam37 linkage couples TOM and SAM complexes into the translocase supercomplex of the mitochondrial outer membrane. Together with previous studies on a late function of Sam37 in precursor maturation at the SAM complex (Chan and Lithgow, 2008; Dukanovic et al., 2009; Becker et al., 2010), we propose that Sam37 plays a dual role in the SAM complex. First, it is directly involved in TOM–SAM coupling. Second, it plays a role in stabilization of the SAM complex. A lack of Sam37 leads to a destabilization of the Sam35–Sam50 subcomplex, in particular at elevated temperature, and delays precursor maturation at SAM and subsequent precursor release (Stojanovski et al., 2007; Chan and Lithgow, 2008; Dukanovic et al., 2009; Becker et al., 2010). Overexpression of Sam35 stabilizes the Sam35–Sam50 subcomplex but does not restore TOM–SAM supercomplex formation in the absence of Sam37. The Sam35–Sam50 subcomplex binds precursor proteins, pointing to distinct functions of the two peripheral SAM subunits in early steps of β -barrel biogenesis: Sam37 promotes the interaction of TOM and SAM complexes, whereas Sam35 cooperates with Sam50 in the recognition of incoming β -barrel precursors (Kutik et al., 2008).

Thus, the TOM–SAM coupling function identified here directly depends on Sam37, whereas the assembly function of Sam37 can, in part, be substituted for by large amounts of small Tom proteins like Tom5 and Tom6 (Dukanovic et al., 2009; Becker et al., 2010). We conclude that Sam37 plays a specific role in formation of the TOM–SAM supercomplex by its binding to Tom22 and additionally participates in the formation of an assembly platform that promotes SAM stability and precursor maturation.

Materials and methods

Yeast strains

The *Saccharomyces cerevisiae* strains used in this study and their genotypes are listed in Table S1. Yeast strains expressing Tom22(K66C)_{His} (4,177), Tom22(Δ IMS)_{His} (Tom22-2_{His}; 4,401), and Tom22(Δ N)_{His} (Tom22(Δ 4-54)_{His}; 4,400) were generated by plasmid shuffling (Becker et al., 2008). The strain *tom22* Δ containing a wild-type copy of *TOM22* in pYEp352 (2,281; Bernard Guiard, Centre national de la recherche scientifique, Gif-sur-Yvette, France; Qiu et al., 2013) was transformed with pFL39 plasmids encoding the Tom22 constructs. Subsequently, cells were selected on media containing 5-fluoroorotic acid to loose pYEp352 encoding the wild-type copy of *TOM22*. To generate a Tom22_{His} *sam37* Δ strain (4,237), genetic information encoding a deca-His tag was chromosomally introduced before the stop codon of *TOM22* in the *sam37* Δ background using a *HIS3* cassette as selection marker. For

overexpression, the coding sequence plus endogenous promoter and terminator of *SAM35* or *TOM6* were integrated into a pRS426 vector. The plasmids were introduced into the yeast strains *sam37* Δ , Tom22_{His}, Tom22_{His} *sam37* Δ , and the corresponding wild-type strain YPH499 using *URA3* as selection marker. Yeast strains were grown on YPG or YPS medium (1% yeast extract, 2% peptone, and 3% glycerol or 2% sucrose, respectively) at 19–24°C. For growth tests, 1 OD of yeast cells was diluted three times in 10-fold serial dilutions in H₂O, dropped on YPG or YPD (1% yeast extract, 2% peptone, 2% glucose) agar plates, and incubated at the indicated temperatures.

Isolation of mitochondria

Mitochondria were isolated by differential centrifugation. Yeast cells were grown to an OD₆₀₀ of 1 in YPG or YPS medium. Cells were harvested by centrifugation at 2,500 g, washed with distilled H₂O, and incubated in 2 ml of 0.1-M Tris, pH 9.4, and 10-mM DTT per gram wet weight of cells for 20 min at 24°C (Meisinger et al., 2006). Cells were reisolated and washed with zymolyase buffer (1.2-M sorbitol and 20-mM K₂HPO₄, pH 7.4). To digest the cell wall, cells were incubated with 4 mg Zymolyase (Seikagaku) per gram cell pellet in 7 ml per gram cell pellet zymolyase buffer for 40 min at 24°C. After a further washing step with zymolyase buffer, the spheroblasts were homogenized on ice in homogenization buffer (0.6-M sorbitol, 10-mM Tris, pH 7.4, 1-mM EDTA, 1-mM PMSF, and 0.2% [wt/vol] BSA) with a glass potter. Cell debris was removed by centrifugation at 2,500 g, and mitochondria were pelleted at 17,000 g. The mitochondrial pellet was washed with SEM buffer (250-mM sucrose, 1-mM EDTA, and 10-mM MOPS/KOH, pH 7.2) and resuspended in SEM buffer. The protein concentration was adjusted to 10 mg/ml. The mitochondria were shock frozen in liquid nitrogen and stored at –80°C.

In vitro import of precursor proteins into isolated mitochondria

To label precursor proteins with [³⁵S]methionine, pGEM-4Z plasmids coding for Tom40, Tom40_{G354A}, Porin_{G276I}, b₂-Tom40, and b₂-Porin were used for coupled in vitro transcription and translation in a cell-free translation system based on rabbit reticulocyte lysates (Promega). Recombinant amounts of Sam37, Tom5, and His₁₀ Tom40 were produced in a cell-free expression system based on wheat germ lysates (5 Prime; Becker et al., 2010). For standard import assays, isolated mitochondria were incubated with up to 10% (vol/vol) of the translation product-containing lysate at 25°C in the presence of 4-mM ATP, 4-mM NADH, 5-mM creatine phosphate, and 100 μ g/ml creatine kinase in import buffer (3% [wt/vol] BSA, 250-mM sucrose, 80-mM KCl, 5-mM MgCl₂, 5-mM methionine, 10-mM MOPS/KOH, pH 7.2, and 2-mM KH₂PO₄; Becker et al., 2010). The import was stopped by transfer on ice. Subsequently, mitochondria were reisolated and washed with SEM buffer. Mitochondrial membranes were solubilized in lysis buffer (50-mM NaCl, 0.1-mM EDTA, 20-mM Tris/HCl, pH 7.4, and 10% [vol/vol] glycerol) containing 1% (wt/vol) digitonin for 15 min on ice. After a clarifying spin, mitochondrial proteins were separated by blue native electrophoresis (Qiu et al., 2013). To dissipate the membrane potential, 8- μ M antimycin, 1- μ M valinomycin, and 20- μ M oligomycin (final concentrations) were added. Two-step import reactions comprised a first import reaction with recombinant amounts of a precursor protein, reisolated of the mitochondria by centrifugation, resuspension of the mitochondrial pellet in import buffer, and a second import reaction with ³⁵S-labeled precursor proteins (Becker et al., 2010).

Affinity purification via nickel–nitrilotriacetic acid (Ni-NTA) chromatography

Purification of Tom22_{His}-containing protein complexes was performed via Ni-NTA chromatography (Qiu et al., 2013). Mitochondria were

lysed with lysis buffer containing 1% (wt/vol) digitonin and 10-mM imidazole for 15 min on ice. In this step, the protein concentration was adjusted to 1 mg/ml. After a clarifying spin, the samples were incubated with Ni-NTA agarose (QIAGEN). Unbound proteins were removed by extensive washing with lysis buffer containing 0.1% (wt/vol) digitonin and 40-mM imidazole. Bound proteins were eluted with lysis buffer containing 0.1% (wt/vol) digitonin and 250-mM imidazole.

To study accumulation of precursor proteins at the SAM complex, recombinant amounts of His -Tom40 were imported into isolated mitochondria. Subsequently, mitochondria were reisolated, solubilized, and subjected to purification via Ni-NTA agarose (the beads were washed with lysis buffer containing 20-mM imidazole and 0.1% digitonin; Becker et al., 2010).

Cross-linking

Chemical cross-linking of mitochondria with the homobifunctional amino-reactive cross-linking reagent disuccinimidyl glutarate (DSG; 1-mM final concentration) or the cysteine-specific cross-linking reagent BMOE (1-mM final concentration; Thermo Fisher Scientific) was performed in SEM buffer for 30 min on ice (Qiu et al., 2013). Cross-linking was quenched by addition of 0.1-M Tris/HCl, pH 7.4, or 50-mM DTT for 15 min on ice, respectively. Mitochondria were reisolated, washed with SEM buffer, and solubilized in lysis buffer containing 1% (wt/vol) SDS and 10-mM imidazole for 5 min at 95°C. Samples were clarified by centrifugation and 1:10 diluted in lysis buffer containing 0.2% (vol/vol) Triton X-100 and 10-mM imidazole before incubation with Ni-NTA agarose. Subsequent washing and elution steps were performed as described in the previous section except that 0.2% Triton X-100 was added as a detergent to the buffers.

Binding assay with Tom22_{CD}

For affinity purification via Tom22_{CD}, the His-tagged Tom22_{CD} was expressed, purified, and coupled to Ni-NTA (Qiu et al., 2013). Mitochondria were lysed with lysis buffer containing 1% (wt/vol) digitonin and 10-mM imidazole for 15 min on ice and incubated with the Tom22_{CD} matrices for 60 min at 4°C. After extensive washing with lysis buffer containing 0.1% (wt/vol) digitonin and 20-mM imidazole, bound proteins were eluted by 500-mM imidazole in lysis buffer containing 0.1% digitonin (Qiu et al., 2013).

β -Signal binding assay

In the β -signal binding assay, the recombinant β -signal of Porin (wild-type or mutant version) fused to a GST tag was expressed in *Escherichia coli*. Bacterial cells were lysed via sonication, and the β -signal was coupled to glutathione Sepharose (GE Healthcare; Kutik et al., 2008). Mitochondria were solubilized in 1% (wt/vol) digitonin in lysis buffer and incubated for 30 min with the matrix. After excessive washing, proteins were eluted via thrombin cleavage overnight at 4°C.

Miscellaneous

For the size estimation of proteins separated by SDS-PAGE, Novex Sharp Prestained (Invitrogen) and SDS-Low Molecular Weight Marker (Sigma-Aldrich) were used. In the case of blue native electrophoresis, a mixture containing thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), lactate dehydrogenase (140 kD), and BSA (66 kD) was used as a molecular weight standard. We used semidry Western blotting to transfer the proteins from the gels to a polyvinylidene fluoride membrane (EMD Millipore). The antibodies used for immunodetection (Table S2) were tested with the corresponding deletion or affinity-tagged mitochondria. The immunosignals were detected by enhanced chemiluminescence and visualized on x-ray films (Amersham Hyperfilm ECL [GE Healthcare]; Medix XBU [Foma]). Subsequently, signals on x-ray films

were digitalized using the ScanMaker 1000XL scanner and SilverFast XRay 6.6.2r1 software (Microtek). We used phosphor imagers (Storm 840 and 865; GE Healthcare) to detect imported ^{35}S -labeled precursor proteins exposed to storage phosphor screens. Import experiments were analyzed by the freeware ImageJ version 1.46r (National Institutes of Health). We used Photoshop CS5 (Adobe) to process images and Illustrator CS5 (Adobe) to compile the figures. We indicated when nonrelevant bands were digitally removed by separating white lines.

Online supplemental material

Fig. S1 shows steady-state levels of proteins and Porin assembly in wild-type and *sam37 Δ* mitochondria. Fig. S2 characterizes strains overexpressing Sam35 or Tom6 in the absence of Sam37. Table S1 provides a list of yeast strains and genotypes. Table S2 provides a list of antibodies used in this study. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201504119/DC1>.

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The authors declare no competing financial interests.

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