Assembly of the Mitochondrial Protein Import Channel
Role of Tom5 in Two-Stage Interaction of Tom40 with the SAM Complex

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INTRODUCTION

The preprotein translocase of the outer mitochondrial membrane (TOM) consists of a central β-barrel channel, Tom40, and six proteins with α-helical transmembrane segments. The precursor of Tom40 is imported from the cytosol by a pre-existing TOM complex and inserted into the outer membrane by the sorting and assembly machinery (SAM). Tom40 then assembles with α-helical Tom proteins to the mature TOM complex. The outer membrane protein Mim1 promotes membrane insertion of several α-helical Tom proteins but also affects the biogenesis of Tom40 by an unknown mechanism. We have identified a novel intermediate in the assembly pathway of Tom40, revealing a two-stage interaction of the precursor with the SAM complex. The second SAM stage represents assembly of Tom5 with the precursor of Tom40. Mim1-deficient mitochondria accumulate Tom40 at the first SAM stage like Tom5-deficient mitochondria. Tom5 promotes formation of the second SAM stage and thus suppresses the Tom40 assembly defect of mim1Δ mitochondria. We conclude that the assembly of newly imported Tom40 is directly initiated at the SAM complex by its association with Tom5. The involvement of Mim1 in Tom40 biogenesis can be largely attributed to its role in import of Tom5.
2008). Sam37 is involved in release of the precursor protein from the SAM complex (Chan and Lithgow, 2008; Dukanovic et al., 2009). The Tom40 precursor then sequentially assembles with the α-helical Tom subunits to form the mature multi-subunit TOM complex. A fourth SAM subunit, the mitochondrial distribution and morphology protein Mdm10, has been found in association with two protein complexes, the SAM complex and the MDM complex that is involved in the formation of mitochondrion-endoplasmic reticulum junctions (Boldogh et al., 2003; Meisinger et al., 2004, 2007; Kornmann et al., 2009; Wideman et al., 2010). SAM-bound Mdm10 promotes late steps of Tom40 assembly and favors association of Tom40 with Tom22 (Meisinger et al., 2004, 2006; Thornton et al., 2010; Yamano et al., 2010).

A further outer membrane protein, the mitochondrial import protein Mim1, transiently interacts with a fraction of SAM complexes (Becker et al., 2008). Mim1 promotes membrane insertion of the majority of α-helical Tom proteins. (i) The precursors of the three small Tom proteins are membrane inserted with the help of Mim1 and subsequently depend on SAM functions for assembly into the TOM complex (Stojanovski et al., 2007a; Becker et al., 2008; Thornton et al., 2010). (ii) The precursors of the receptors Tom20 and Tom70 are inserted into the outer membrane with the help of Mim1, but independently of SAM, and then assemble with a TOM core complex consisting of Tom40, Tom22, and the small Tom proteins (Meisinger et al., 2004; Milenkovic et al., 2004; Stojanovski et al., 2007a; Becker et al., 2008; Hulett et al., 2008; Popovic-Celeketic et al., 2008). (iii) Additionally, Mim1-deficient mitochondria are also impaired in Tom40 assembly. Tom40 assembly stages following the SAM complex were inhibited in the mutant mitochondria (Ishikawa et al., 2004; Waizenegger et al., 2005; Becker et al., 2008). Although the involvement of Mim1 in Tom40 assembly was reported before its role in insertion of α-helical proteins was found, it is unknown how Mim1 affects the biogenesis of the β-barrel precursor.

Each of the small Tom proteins was found to be involved in the assembly pathway of Tom40. Tom5 and Tom6 were reported to associate with the Tom40 precursor after its release from the SAM core complex, forming an assembly intermediate that associates with further Tom subunits (Model et al., 2001; Wiedemann et al., 2003). Dukanovic et al. (2009) showed that Tom6 genetically and functionally interacts with Sam37; however, they did not observe a direct interaction between Tom6 and Sam37. The currently available results indicate that Tom6 stabilizes Tom40 precursor molecules and promotes their association with Tom22 (Alconada et al., 1995; Dekker et al., 1998; Dembowski et al., 2001; Dukanovic et al., 2009). In contrast, Tom7 was found to delay Tom40 assembly by promoting a release of Mdm10 from the SAM complex (Model et al., 2001; Meisinger et al., 2006). The exact molecular mechanisms underlying the function of small Tom proteins in Tom40 assembly have not been clarified.

For this study, we have addressed the role of small Tom proteins in the interaction of Tom40 with the SAM complex. We dissected the interaction of the Tom40 precursor with SAM into two stages. In a first stage, Tom40 binds to the SAM core complex. Tom5 is critical for formation of the second stage by assembling with newly synthesized Tom40 directly at the SAM complex. Interestingly, mitochondria lacking Mim1 accumulate the Tom40 precursor at the first SAM stage (i.e., before the assembly with Tom5). The Tom40 assembly defect of mim1Δ mitochondria was indeed suppressed by Tom5. Our results show that small Tom proteins assemble with newly synthesized Tom40 already at the SAM complex and explain the influence of Mim1 on Tom40 assembly by its role in the import of small Tom proteins.

### MATERIALS AND METHODS

#### Yeast Strains

The S. cerevisiae mutant strains tomΔ, tomΔ, tomΔ, sam37Δ, mdm10Δ, mim1Δ, and the corresponding wild-type strains have been described before (Alconada et al., 1995; Herrlinger et al., 1996; Dietmeier et al., 1997; Wiedemann et al., 2003; Meisinger et al., 2004; Becker et al., 2008). The viability of the double deletions sam37Δ tom5Δ, sam37Δ tom1Δ, and sam37Δ tom7Δ was assayed by a plasmid shuffling approach. The open reading frame of SAM37 was cloned into the vector pREP5/T25 for the control of ACT1 promoter activity and a terminator. A yeast strain with a disruption of the open reading frame of SAM37 by the LEU2-Marker was transformed with pYPE352 encoding SAM37. Clones were selected using the URA3 marker of the plasmid. After this, a kanamycin resistance cassette was introduced in the open reading frames of TOM5, TOM6, or TOM7. Finally, the plasmid encoding SAM37 was eliminated by growth on plates containing 5-fluoroorotic acid (5-FOA).

For generation of overexpression mutants, the open reading frames of TOM5, TOM6, TOM7, MIM1, or SAM37 were introduced in a pYPE352 vector. Subsequently, the yeast strains tomΔ and samΔ were transformed and clones were selected by the URA3 marker of the plasmid.

#### Protein Import into Mitochondria

Mitochondria were isolated by differential centrifugation according to standard procedures (Stojanovski et al., 2007b). The protein concentrations were adjusted to 10 mg/ml in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS/KOH [pH 7.2]). Mitochondria aliquots were shock-frozen in liquid nitrogen and stored at −80°C. For in vitro import assays, 15S-labeled proteins were synthesized using pCMA24-based constructs as template for coupled in vitro transcription and translation (Promega, Madison, WI). In a standard import assay, 50 μg mitochondria (protein amount) were incubated with the radiolabeled proteins in the presence of 2 mM NADH, 2 mM ATP, 5 mM methionine, 5 mM creatine phosphate, and 100 μg/ml creatine kinase in import buffer (3% [wt/vol] BS5A, 250 mM sucrose, 80 mM KCl, 5 mM MgCl2, 10 mM MOPS/KOH [pH 7.2], 2 mM KH2PO4). Transfer on ice stopped the import reaction. Mitochondria were resuspended, washed with SEM buffer, and lysed with 1% (wt/vol) digitonin in lysis buffer (20 mM Tris/HCl pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% [vol/vol] glycerol) at a protein concentration of 1 mg/ml for 15 min on ice. After a clarifying spin, mitochondrial lysates were loaded onto a blue native gel as described (Dekker et al., 1998; Ryan et al., 2001; Stojanovski et al., 2007b). Protein complexes were visualized by digital autoradiography. For a two-step import assay, chemical amounts of the proteins were generated in vitro using a wheat germ based translation system (5 Prime, Hamburg, Germany). These proteins were imported into isolated mitochondria for 40 min at 25°C. Subsequently, mitochondria were resuspended and washed with SEM buffer. The import of radiolabeled precursors into these mitochondria was performed according to the conditions described above.

#### Antibody Shift Assays

For antibody shift assays, radiolabeled proteins were imported into isolated mitochondria. After washing, mitochondria were incubated in SEM buffer with the indicated antibodies for 30 min on ice. Mitochondria were resuspended and lysed with 1% (wt/vol) digitonin in lysis buffer (20 mM Tris/HCl pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% [vol/vol] glycerol) at a protein concentration of 1 mg/ml for 15 min on ice. After a clarifying spin, mitochondrial lysates were loaded onto a blue native gel as described (Dekker et al., 1998; Ryan et al., 2001; Stojanovski et al., 2007b). Protein complexes were visualized by digital autoradiography. For a two-step import assay, chemical amounts of the proteins were generated in vitro using a wheat germ based translation system (5 Prime, Hamburg, Germany). These proteins were imported into isolated mitochondria for 40 min at 25°C. Subsequently, mitochondria were resuspended and washed with SEM buffer. The import of radiolabeled precursors into these mitochondria was performed according to the conditions described above.

#### Miscellaneous

Western blotting transfer onto PVDF membranes and immunodecoration were performed according to standard procedures. Enhanced chemiluminescence (GE Healthcare, Piscataway, NJ) was used for the detection of the antibodies.

### RESULTS

#### Genetic Interaction of TOM5 and SAM37

Yeast cells lacking SAM37 show a growth defect at elevated temperature (Gratzer et al., 1995; Dukanovic et al., 2009). Dukanovic et al. (2009) reported that expression of TOM6, but neither TOM5 nor TOM7, from a high copy number plasmid (pRS426) suppressed the growth defect of sam37Δ cells. When we expressed the small TOM genes via the high copy number plasmid pYPE352 (with a MET25 promoter), we unexpectedly found that overexpression of TOM5 suppressed the growth defect of sam37Δ cells as efficiently as
TOM40 growth when the plasmid encoding the wild-type copy of SAM37 and TOM5 and SAM37 leads to synthetic lethality. For comparison, we also generated the double mutants sam37Δ tom6Δ and sam37Δ tom7Δ in our strain background and observed a strong synthetic growth defect of sam37Δ tom6Δ cells, whereas sam37Δ tom7Δ cells grew like the sam37Δ single deletion mutant (Figure 1B) in agreement with Dukanovic et al. (2009). These results demonstrate that not only TOM6 but also TOM5 shows a strong genetic interaction with SAM37.

Tom5 Suppresses the Defect of Sam37 in Tom40 Assembly

To explore the molecular mechanisms behind these phenotypes we wanted to obtain biochemical evidence for a relation of Tom5 to Sam37 (i.e., we asked whether Tom5 was able to suppress protein sorting defects in sam37Δ mitochondria). A detailed functional analysis of outer membrane protein biogenesis can be performed by an in organello system using purified mitochondria and precursor proteins that are synthesized and radiolabeled in reticulocyte lysate in the presence of [35S]methionine (Ryan et al., 2001; Stojanovski et al., 2007b). However, reticulocyte lysates typically synthesize proteins in small radiochemical amounts that are not sufficient to resolve mutant defects on a biochemical level. The expression of mitochondrial membrane proteins in E. coli cells produces large chemical amounts but usually leads to aggregation in inclusion bodies. Only few mitochondrial membrane proteins have been successfully extracted from inclusion bodies in a denatured but transport-competent form and imported into mitochondria. We thus established a system to efficiently synthesize mitochondrial proteins in vitro using a wheat germ–based translation system. To test whether Tom5 synthesized by this system was imported into mitochondria and assembled into the TOM complex, we used mitochondria that were isolated from a yeast strain lacking TOM5. When mitochondria are lysed with the nonionic detergent digitonin and separated by blue native electrophoresis, the TOM complex migrates at ~450 kDa (Dekker et al., 1998). On import of chemical amounts of Tom5, the mobility of the TOM complex was fully shifted to wild-type conditions (Figure 2A, lane 3), demonstrating that Tom5 synthesized in the wheat germ system was import-competent and assembled into the TOM complex.

Mitochondria were isolated from sam37Δ cells and incubated with Tom5, Tom6, or Tom7 synthesized in the wheat germ system. On reisolation of the mitochondria, radiola-
Intermediates of \([35S]Tom40\) consisted of two distinct bands that were separated on high-resolution blue native gels and observed that the SAM-Ia stage of the TOM complex, the formation of SAM, Int-II, and mature TOM complex is impaired, and the precursor of Tom40 is found in association with a smaller SAM complex that lacks Sam37 (Wiedemann et al., 2003; Waizenegger et al., 2004; Milenkovic et al., 2004; Chan and Lithgow, 2008; Dukanovic et al., 2009). When chemical amounts of Tom5 were imported into mitochondria, the formation of SAM-Ia, intermediate II and mature TOM complex was efficiently restored (Figure 2B, lanes 1 and 2) (Model et al., 2003; Wiedemann et al., 2003; Waizenegger et al., 2004; Chan and Lithgow, 2008; Dukanovic et al., 2009). When chemical amounts of Tom5 were imported into mitochondria, SAM-Ia and SAM-Ib, respectively (Figure 3A, lanes 1–3). In the SAM-Ia stage, Tom5 was present in the SAM-Ib intermediate, whereas SAM-Ib without importing additional amounts of endogenous Tom5 was present in the SAM-Ib intermediate. Tom5 Flag accelerated the formation of the SAM-Ib intermediate, whereas Tom7 Flag had no effect (Figure 3B, lanes 2, 3). The addition of anti-Flag antibodies revealed that the mobility of accumulated Tom5 was shifted to larger molecular masses by anti-Flag (Figure 3B, lane 3), demonstrating that the Tom40 precursor was associated with Tom5 Flag. To test whether endogenous Tom5 was present in the SAM-Ib intermediate, we performed a short-term import of \([35S]Tom40\) into wild-type mitochondria without importing additional amounts of Tom5. Antibodies directed against Tom5 were added to the mitochondria. Blue native electrophoresis showed that anti-Tom5 affected SAM-Ib and intermediate II but not SAM-Ia (Figure 4C, lane 2). The addition of control antibodies raised against the outer membrane protein Fis1 did not affect the mobility of Tom40 intermediates (Figure 4C, lane 3). We conclude that Tom5 is part of the SAM-Ib intermediate.

Taken together, the precursor of Tom40 interacts with the SAM complex in two stages, SAM-Ia and SAM-Ib. Tom5 associates with the Tom40 precursor at the SAM complex, thus forming intermediate SAM-Ia.

Tom6 Partially Substitutes for Tom5 in Maturation of Tom40

We asked whether the two other small Tom proteins also affected the formation of Tom40 intermediates at the SAM

**Two-Stage Interaction of Tom40 with the SAM Complex Involves Tom5**

It has been shown that Tom5 interacts with the precursor of Tom40 in the assembly intermediate II and that mitochondria lacking Tom5 accumulate the Tom40 precursor at the SAM complex (Model et al., 2003; Wiedemann et al., 2003). We used high-resolution blue native gels and observed that the SAM intermediate of \([35S]Tom40\) consisted of two distinct bands that have not been characterized so far. We termed these bands SAM-Ia and SAM-Ib, respectively (Figure 3A, lanes 1–3). In the SAM-Ia stage, Tom5 was present in the SAM-Ib intermediate, whereas Tom7 was absent. We tested whether the two other small Tom proteins also affected the formation of Tom40 intermediates (Figure 4C, lane 2). We conclude that Tom5 is part of the SAM-Ib intermediate.

**Figure 3.** Tom5 is involved in the two-stage interaction of the Tom40 precursor with the SAM complex. (A) \([35S]Tom40\) was imported into wild-type (WT) and tom5Δ yeast mitochondria for the indicated periods. Mitochondria were mock treated or incubated with Flag–Tom5. Subsequently, the assembly of \([35S]Tom40\) was analyzed by blue native electrophoresis and autoradiography.
complex. Mitochondria lacking Tom6 efficiently generated the SAM-Ia intermediate but were partially impaired in the formation of SAM-Ib and the assembly of Tom40 into the TOM complex (Figure 5A). In contrast, mitochondria lacking Tom7 efficiently generated both SAM intermediates (Figure 5B) (integration of [35S]Tom40 into the mature TOM complex occurred with higher efficiency in tom7Δ mitochondria than in wild-type mitochondria as previously reported [Meisinger et al., 2006]). Tom7 functions in an antagonistic manner to Mdm10 that promotes assembly of Tom40 into the TOM complex (Meisinger et al., 2004, 2006; Yamano et al., 2010). We thus also analyzed mdm10Δ mitochondria and observed that the SAM-Ib intermediate of [35S]Tom40 was generated (Figure 5C) ([35S]Tom40 accumulated in assembly intermediate II as reported [Meisinger et al., 2004, 2006]). Thus, of the three mutant mitochondria, tom6Δ, tom7Δ, and mdm10Δ, only tom6Δ mitochondria were (partially) impaired in formation of the new SAM-Ib intermediate.

To address whether Tom6 may play a role in formation of the SAM-Ib intermediate, we imported chemical amounts of Tom6 into isolated mitochondria before importing the [35S]Tom40 precursor. The import of Tom6 indeed promoted the formation of the SAM-Ib stage (Figure 6A, lanes 5 and 6). Chemical amounts of Flag-tagged Tom6 similarly accelerated the formation of SAM-Ib (Figure 6B, lane 2). Anti-Flag antibodies shifted part of the SAM-Ib intermediate on blue native gels (Figure 6B, lane 3), indicating that Tom6 associated with the precursor of Tom40 at the SAM-Ib stage. We thus asked whether Tom6 could replace the function of Tom5 in Tom40 maturation. We imported chemical amounts of small Tom proteins into tom5Δ mitochondria and analyzed the assembly stages of [35S]Tom40 (Figure 6C). Chemical amounts of Tom6 promoted the assembly of Tom40 into the TOM complex, although with considerably lower efficiency than Tom5 did (Figure 6C, compare lane 6 to lane 8). Moreover, Tom6 supported the formation of SAM-Ib in tom5Δ mitochondria only with low efficiency (Figure 6C, lane 7), in contrast to the efficient promotion of SAM-Ib formation in wild-type mitochondria (Figure 6, A and B), indicating that the presence of Tom5 is required for an efficient formation of SAM-Ib. Chemical amounts of Tom7 had no stimulatory effect on Tom40 assembly (Figure 6C, lanes 9 and 10). In agreement with these biochemical results, overexpression of Tom6, but not Tom7, partially rescued growth of tom5Δ cells at elevated temperature (Figure 6D).

Taken together, these results show that Tom6 can partially substitute for Tom5 in Tom40 assembly, yet Tom5 is the main factor required for generation of the SAM-Ib intermediate. Tom5 and Tom6 can associate with the precursor of Tom40 at the SAM-Ib stage.

Tom5 Suppresses the Defect of mim1Δ Mitochondria in Tom40 Assembly

Using the new assembly intermediate of Tom40 and mim1Δ mitochondria, we made a surprising observation. Mitochondria lacking Mim1 were not only impaired in the formation of Tom40 assembly after the Sam complex (Ishikawa et al., 2004; Waizenegger et al., 2005; Becker et al., 2008) but also in the generation of the SAM-Ib intermediate (Figure 7, lanes 3 and 4). [35S]Tom40 mainly accumulated in the SAM-Ia stage, and only little SAM-Ib was generated. Strikingly, chemical amounts of Tom5 imported into mim1Δ mitochondria efficiently restored the assembly of Tom40 (Figure 7, lanes 5 and 6). Chemical amounts of Tom6 moderately stimulated the generation of SAM-Ib, whereas Tom7 did not promote Tom40 assembly at all (Figure 7, lanes 7–10). We conclude that Tom5 can suppress the Tom40 assembly defect of mim1Δ mitochondria.
DISCUSSION

We have identified a novel intermediate in the biogenesis of the protein import channel of the mitochondrial outer membrane. On its assembly pathway, the precursor of the β-barrel protein Tom40 has to associate with several α-helical Tom proteins, first the small Tom proteins and then the Tom receptors (Model et al., 2001; Wiedemann et al., 2003; Meisinger et al., 2004; Becker et al., 2008; Hulett et al., 2008; Popov-Celeketic et al., 2008; Dukanovic et al., 2009; Thornton et al., 2010). To date it has been assumed that the α-helical Tom proteins associate with the Tom40 precursor after its release from the SAM complex. We report that Tom5 plays a critical role in Tom40 assembly already at the SAM complex. The precursor of Tom40 stably interacts with SAM in the absence of Tom5, however further progression of assembly strongly depends on Tom5. By high-resolution blue native electrophoresis we could dissect two assembly stages of Tom40 at the SAM complex, the first one, SAM-Ia, representing the Tom5-independent binding of Tom40 to SAM, whereas the second stage, SAM-Ib, represents the assembly of Tom5 with Tom40 at the SAM complex.

The mitochondrial outer membrane contains only three proteins that are essential for cell viability under all growth conditions tested: Tom40 and the two core components Sam50 and Sam35 of the SAM complex (Baker et al., 1990; Kozjak et al., 2003; Paschen et al., 2003; Gentle et al., 2004; Ishikawa et al., 2004; Milenkovic et al., 2004; Waizenegger et al., 2004). Yeast cells lacking Tom5 are inviable at elevated temperature yet are viable at lower temperature. We asked whether and which component may be able to substitute for Tom5. We observed that overexpression of Tom6, but not Tom7, partially suppressed the growth defect of tom5Δ yeast cells. Because the small Tom proteins are subunits of the mature TOM complex, it may be argued that this genetic relation is only caused by their function in the TOM complex. However, a genetic interaction of TOM5 as well as TOM6 with SAM is indicated by two lines of evidence: the growth defect of sam37Δ cells is suppressed by overexpression of Tom5, as well as by overexpression of Tom6; and a double deletion of TOM5 and SAM37 as well as a double deletion of TOM6 and SAM37 cause strong synthetic growth defects, whereas no genetic interaction be-

Figure 6. Tom6 partially substitutes for Tom5 in the assembly of Tom40. (A) Chemical amounts of Tom5 or Tom6 were imported into wild-type (WT) mitochondria. After reisolation of the mitochondria, 35S-labeled Tom40 was imported, and the mitochondria were lysed and analyzed by blue native electrophoresis and autoradiography. (B) Chemical amounts of Tom6Flag were imported into wild-type mitochondria. 35S-labeled Tom40 was imported into the reisolated mitochondria, and anti-Flag antibodies were added to the lysed mitochondria. The assembly of Tom40 was analyzed by blue native electrophoresis. (C) 35S-labeled Tom40 was imported into wild-type and tom5Δ mitochondria that had been preincubated with chemical amounts of the indicated small Tom proteins. (D) Overexpression of the indicated proteins was performed in a tom5Δ yeast strain. The growth of the indicated strains was analyzed on selective medium containing glycerol and ethanol as energy source.

Figure 7. Chemical amounts of Tom5 compensate for the loss of Mim1 in the assembly of Tom40. 35S-labeled Tom40 was imported into mim1Δ mitochondria that had been preincubated with chemical amounts of the indicated small Tom proteins. The import of Tom40 was analyzed by blue native electrophoresis and autoradiography.
between TOM7 and SAM37 was observed (Dukanovic et al., 2009; this study). With the in organello assembly assay, we found that tom6Δ mitochondria were partially impaired in formation of the SAM-Ib stage, resembling a milder form of the defect of tom5Δ mitochondria, suggesting that Tom6 may indeed be involved in an early stage of Tom40 biogenesis at the SAM complex.

To directly define the functions of individual small Tom proteins, we established a combined genetic-biochemical approach. Mitochondrial protein sorting defects of yeast deletion mutants were complemented by synthesizing small Tom proteins in chemical amounts and importing them into isolated mitochondria. With this approach we could show that Tom5 as well as Tom6 promoted the assembly pathway of Tom40 at the SAM complex, whereas Tom7 had no stimulatory effect. By antibody shift analysis, we demonstrated that Tom5 and Tom6 associated with the precursor of Tom40 at the SAM-Ib stage, thus providing a direct biochemical explanation for the genetic connections. Tom5 plays the major role in Tom40 maturation at the SAM complex for the following reasons. First, the conversion of SAM-Ia to SAM-Ib is more severely inhibited in tom5Δ than in tom6Δ mitochondria. Second, chemical amounts of Tom6 strongly stimulate the formation of SAM-Ib when Tom5 is present in wild-type amounts, but only partially rescue Tom40 assembly when Tom5 is absent.

The new assembly intermediate provided the possibility to address the role of Mim1 in Tom40 assembly. We found that mitochondria lacking Mim1 displayed a similar defect in assembly of the Tom40 precursor as mitochondria lacking Tom5 (i.e., accumulation of the precursor at the first stage of SAM interaction). Because Mim1 is required for the efficient import and membrane insertion of small Tom proteins (Becker et al., 2008; Thornton et al., 2010), we speculated that the role in import of small Tom proteins may explain the requirement of Mim1 for the assembly pathway of Tom40. Indeed, import of chemical amounts of Tom5 fully suppressed the Tom40 assembly defect of mim1Δ mitochondria, whereas Tom6 partially suppressed the defect—in line with the role of these small Tom proteins in Tom40 maturation at the SAM complex. We conclude that Mim1 is not directly needed for the assembly stages of Tom40 but functions via the import of small Tom proteins.

After the Mim1-dependent membrane insertion, the precursors of small Tom proteins depend on SAM functions for assembly into the TOM complex (Stojanovski et al., 2007a; Becker et al., 2008; Thornton et al., 2010). In case of Tom6, an interaction of the radiolabeled precursor with the SAM complex was observed. The Tom6 precursor associated with a module of pre-existing Tom40-Tom5 at the SAM complex; however, due to the transient nature of the intermediate the Tom6-SAM interaction was observed in only low abundance (Thornton et al., 2010) and thus it remained open whether this assembly at the SAM complex was only a special pathway for the precursor of Tom6 or whether it represented a main mechanism for the biogenesis of further Tom proteins. We show here that the two stages of Tom40 interaction with the SAM complex represent main import stages in the biogenesis pathway of this essential β-barrel precursor and conclude that the assembly of Tom40 with small Tom proteins represents an important function of the SAM complex.

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