doi:10.1016/j.jmb.2010.11.002 J. Mol. Biol. (2011) 405, 113–124

Contents lists available at www.sciencedirect.com

Journal of Molecular Biology

journal homepage: http://ees.elsevier.com.jmb

Biogenesis of Mitochondria: Dual Role of Tom7 in Modulating Assembly of the Preprotein Translocase of the Outer Membrane

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Received 26 September 2010; received in revised form 25 October 2010; accepted 2 November 2010
Available online 6 November 2010

Edited by J. Karn

Keywords:
Mdm10;
Saccharomyces cerevisiae;
SAM complex;
Sam50;
TOM complex

Biogenesis of the translocase of the outer mitochondrial membrane (TOM complex) involves the assembly of the central β-barrel forming protein Tom40 with six different subunits that are embedded in the membrane via α-helical transmembrane segments. The sorting and assembly machinery (SAM complex) of the outer membrane plays a central role in this process. The SAM complex mediates the membrane integration of β-barrel precursor proteins including Tom40. The small Tom proteins Tom5 and Tom6 associate with the precursor of Tom40 at the SAM complex at an early stage of the assembly process and play a stimulatory role in the formation of the mature TOM complex. A fraction of the SAM components interacts with the outer membrane protein mitochondrial distribution and morphology protein 10 (Mdm10) to form the SAM–Mdm10 machinery; however, different views exist on the function of the SAM–Mdm10 complex. We report here that the third small Tom protein, Tom7, plays an inhibitory role at two distinct steps in the biogenesis of the TOM complex. First, Tom7 plays an antagonistic role to Tom5 and Tom6 at the early stage of Tom40 assembly at the SAM complex. Second, Tom7 interacts with Mdm10 that is not bound to the SAM complex, and thus promotes dissociation of the SAM–Mdm10 complex. Since the SAM–Mdm10 complex is required for the biogenesis of Tom22, Tom7 delays the assembly of Tom22 with Tom40 at a late stage of assembly of the TOM complex. Thus, Tom7 modulates the biogenesis of topologically different proteins, the β-barrel forming protein Tom40 and Tom22 that contains a transmembrane α-helix.

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Abbreviations used: Mdm, mitochondrial distribution and morphology; Mim1, mitochondrial import protein 1; SAM, sorting and assembly machinery; TOM, translocase of outer mitochondrial membrane.

Introduction

Most mitochondrial proteins are synthesized on cytosolic ribosomes as precursor proteins and have to be imported into the organelle. The translocase of the outer mitochondrial membrane (TOM complex) forms the entry gate for most mitochondrial proteins and is essential for cell survival.[1–4] The TOM

complex consists of seven different subunits. The β-barrel protein Tom40 forms the protein-conducting channel and associates with six components with a single α-helical transmembrane segment.[5–10] The receptors Tom20 and Tom70 recognize the majority of incoming precursor proteins.[11–16] Precursor proteins are subsequently delivered to the central receptor Tom22, which also serves as docking site for Tom20 and Tom70.[17,18] The three small Tom proteins are important for function and stability of the TOM complex.[19–26] Tom5 is also involved in the transfer of precursor proteins to the translocation pore.[21] Deletion of Tom6 and Tom7 affects the stability of the TOM complex in an antagonistic manner. In the absence of Tom6, the TOM complex is destabilized, whereas deletion of Tom7 stabilizes the interactions of the TOM subunits.[19,20,22] Based on these observations, it was concluded that Tom6 and Tom7 regulate the integrity of the TOM complex.

The precursors of all TOM components are encoded by nuclear genes, synthesized on cytosolic ribosomes, and imported into mitochondria. The sorting and assembly machinery (SAM complex) of the outer membrane fulfills an important role in the import and association of the β-barrel protein Tom40 with other Tom proteins containing α-helical transmembrane segments.[27–29] Precursors of β-barrel proteins are first transported across the outer membrane via preexisting TOM complexes.[30] Subsequently, small TIM chaperone complexes of the intermembrane space assist in the transfer of the precursors to the SAM complex, which catalyzes the membrane integration of the precursor proteins.[31,32] The SAM core complex consists of three subunits: a central component, Sam50 (also termed Omp85/Tob55), and two peripheral components, Sam35 (Tom38/Tob38) and Sam37 (Mas37).[30,33–38] Sam50 forms a β-barrel structure and contains an N-terminal polypeptide translocation associated domain (POTRA).[33–35] The structural features of Sam50 are characteristic for the conserved Omp85 (BamA) protein family, which plays a central role in the biogenesis of β-barrel proteins of the outer membrane of Gram-negative bacteria, mitochondria, and plastids.[1,39–41] Although Sam50 is the central component of this machinery, its exact molecular mechanism remains to be investigated. Sam35 is involved in precursor binding, whereas Sam37 promotes the release of precursors from the SAM complex.[42–44] Sam50 and Sam35 are essential for yeast survival, indicating the importance of the biogenesis of β-barrel precursors.[33–37]

Recently, it was shown that the SAM complex associates with different partner proteins to mediate the biogenesis of Tom proteins with α-helical transmembrane segments.[45–48] The SAM complex transiently interacts with mitochondrial import protein 1 (Mim1), which supports the insertion of several α-helical proteins, including the small Tom proteins, into the outer membrane.[46,47] Tom5 and Tom6 then associate with the precursor of Tom40 at an early assembly step that directly occurs at the SAM complex.[47,48] Tom40 with bound Tom5 and Tom6 proceeds to later assembly steps towards formation of the mature TOM complex.[49] The role of Tom7 in TOM biogenesis during these steps is not yet defined.

A fraction of the SAM_{core} complexes associate with a further β-barrel protein of the outer membrane, mitochondrial distribution and morphology protein 10 (Mdm10), to form the SAM–Mdm10 complex.[47,49–53] Mdm10 plays a dual role as it is also present in a second protein complex together with Mdm12, Mdm34 (Mmm2), and Mmm1.[51,54–56] This MDM complex was originally found to regulate mitochondrial morphology.[54] Recent work showed a role of the MDM complex in tethering of the endoplasmic reticulum to mitochondria, and thus it was also named ERMES (endoplasmic reticulum–mitochondria encounter structure).[56] Currently, the function of the SAM–Mdm10 complex in assembly of the TOM complex is a subject of debate. Two different models have been proposed. On one hand, it was suggested that Mdm10 mediates release of the Tom40 precursor from the SAM complex.[53] This model is based on the observation that in an Mdm10-overexpressing strain, the interaction of β-barrel precursors with the SAM complex is impaired, which implies that Mdm10 competes with β-barrel precursors for binding to the SAM_{core} complex.[53] On the other hand, it was proposed that the SAM–Mdm10 complex functions in late assembly steps of the TOM complex.[47,49,50] The precursor of Tom22 was found to bind to the SAM–Mdm10 complex, and mitochondria lacking Mdm10 accumulated the precursor of Tom40 in an intermediate form that was released from the SAM_{core} complex.[47–50] Together with the observation that the association of Tom40 with Tom22 is crucial for the formation of the mature TOM complex,[17] these findings favor the idea that Mdm10 is involved in late steps of TOM complex formation. Interestingly, the formation of the SAM–Mdm10 complex is increased in mutant mitochondria lacking Tom7, leading to a stimulation of Tom40 assembly.[50,52] The molecular mechanism of how Tom7 affects the SAM–Mdm10 complex is not understood.

In this study, we analyzed the function of Tom7 in the assembly of the TOM complex. Surprisingly, we found that Tom7 plays a dual role. First, it delays the assembly of Tom40 at the SAM complex by functioning in an antagonistic manner to Tom5 and Tom6. Second, Tom7 binds to Mdm10 and promotes dissociation of Mdm10 from the SAM–Mdm10 complex, leading to an impaired assembly of Tom22. Thus, Tom7 plays a regulatory role at two stages of assembly of the TOM complex by retarding assembly of Tom40 as well as Tom22.

Results and Discussion

Tom7 impairs the biogenesis of Tom40

It has been reported that Tom5 and Tom6 bind to the precursor of Tom40 at the SAM complex and play a stimulatory role in the assembly of Tom40.[48] We asked whether Tom7 is also involved in this process. We synthesized large (chemical) amounts of Tom7 precursor in a wheat germ-based translation system (the usual *in vitro* translation system, rabbit reticulocyte lysate, typically produces only small, radiochemical amounts of mitochondrial precursor proteins). It has been shown that the precursors of several outer membrane proteins produced in the wheat germ system are competent for import into mitochondria and assemble into functional protein machineries.[48] To confirm the import competence of the Tom7 precursor, we imported the precursor into mutant mitochondria lacking Tom7. The mitochondria were lysed with the nonionic detergent digitonin, and the TOM complex was analyzed by blue native electrophoresis (Fig. 1a). In the absence of Tom7, the TOM complex migrates at a lower molecular mass on the native gel than the wild-type TOM complex (Fig. 1a, lanes 1 and 2).[22] After import of *in vitro* synthesized Tom7, the native mobility of the TOM complex was fully restored to wild-type behavior (Fig. 1a, lane 3), demonstrating that chemical amounts of Tom7 were efficiently imported and assembled. We used this approach to study the impact of chemical amounts of Tom7 on the biogenesis of Tom40. The assembly steps of the precursor of Tom40 in the outer mitochondrial membrane can be monitored by blue native electrophoresis upon importing ^{35}S-labeled Tom40.[30,57] At early time points, the Tom40 precursor binds to the SAM complex (stage SAM-Ia; Fig. 1b, lanes 1 and 2), followed by association of Tom5 (stage SAM-Ib; Fig. 1b, lanes 1–3).[48] At later time points, Tom40 and Tom5 are present in a second smaller intermediate (stage Int-II; Fig. 1b, lanes 2 and 3) before they assemble into a mature TOM complex (Fig. 1b, lane 3).[30] When chemical amounts of Tom7 were first imported into mitochondria, however, the precursor of Tom40 was mainly arrested at the SAM-Ia stage, whereas the formation of the subsequent assembly stages, SAM-Ib, intermediate II, and mature TOM, was strongly inhibited (Fig. 1b, lanes 4–6). This suggested that chemical amounts of Tom7 impaired the formation of the SAM-Ib stage, which involves the association of Tom5 with the precursor of Tom40. Since the SAM machinery mediates the biogenesis of β-barrel proteins in general, we wondered whether Tom7 impaired the assembly of other β-barrel proteins as well. We thus studied the import of the β-barrel protein porin into mitochondria in the presence of chemical amounts of Tom7. Import and assembly of porin were not blocked by high levels of Tom7 (Fig. 1c). These results suggest a role of Tom7 in modulating early steps of the assembly pathway of Tom40.

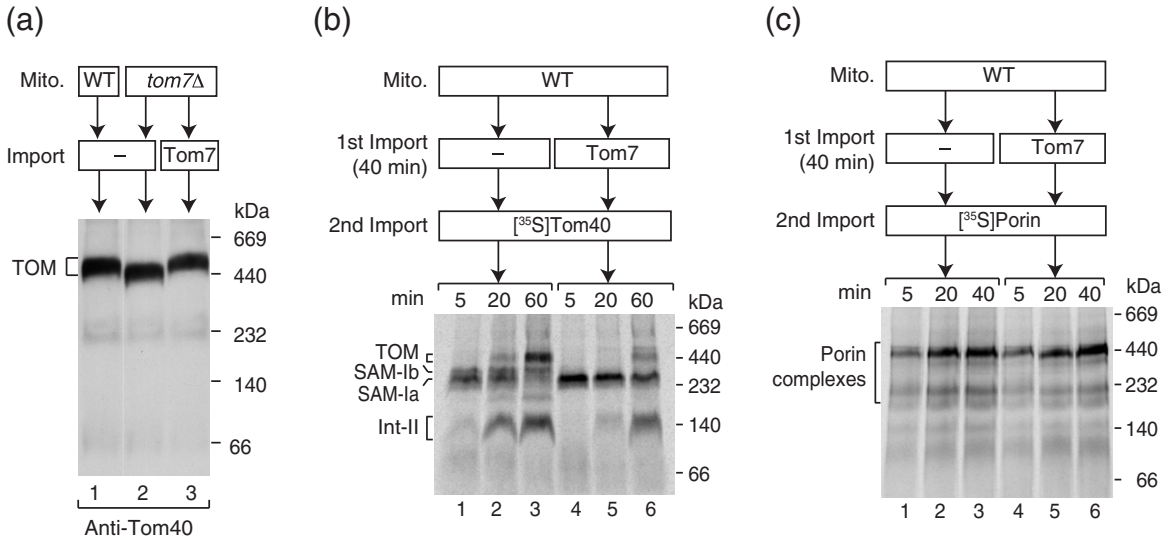

Fig. 1. Tom7 retards maturation of Tom40 at the SAM complex. (a) Chemical amounts of Tom7 were synthesized with a wheat germ-based translation system and imported into wild-type (WT) and *tom7*Δ yeast mitochondria. Mitochondria were solubilised with digitonin, and protein complexes were separated by blue native electrophoresis. The TOM complex was detected using an antiserum against Tom40. (b and c) Chemical amounts of Tom7 were imported into wild-type mitochondria for 40 min at 25 °C. After reisolation of mitochondria, ^{35}S-labeled Tom40 or porin was imported for the indicated time periods. Protein complexes were separated by blue native electrophoresis and analyzed by digital autoradiography.

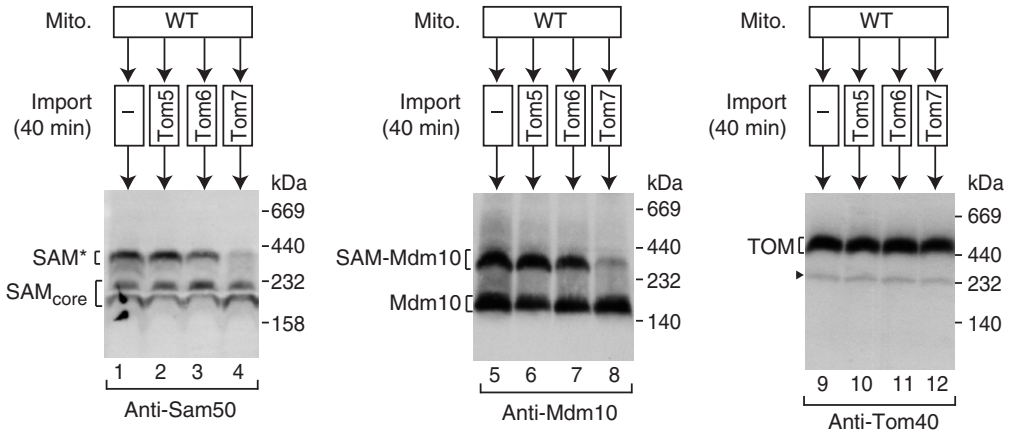

Fig. 2. Tom7 induces dissociation of the SAM–Mdm10 complex. Chemical amounts of Tom5, Tom6, and Tom7 were imported into isolated yeast wild-type mitochondria for 40 min at 25 °C. Mitochondria were solubilised with digitonin and subjected to blue native electrophoresis. Protein complexes were detected using antibodies against Sam50, Mdm10, and Tom40. Arrowhead, nonspecific band.

Tom7 interacts with Mdm10 and promotes formation of a SAM-free form of Mdm10

We asked if high levels of small Tom proteins affected the stability of the SAM complex and/or TOM complex. To address this issue, we imported chemical amounts of Tom5, Tom6, or Tom7 and analyzed the TOM and SAM complexes by blue native electrophoresis (Fig. 2). Different forms of SAM complexes have been described: the SAM_{core} complex containing Sam35, Sam37, and Sam50 migrates at ~200 kDa, whereas larger SAM

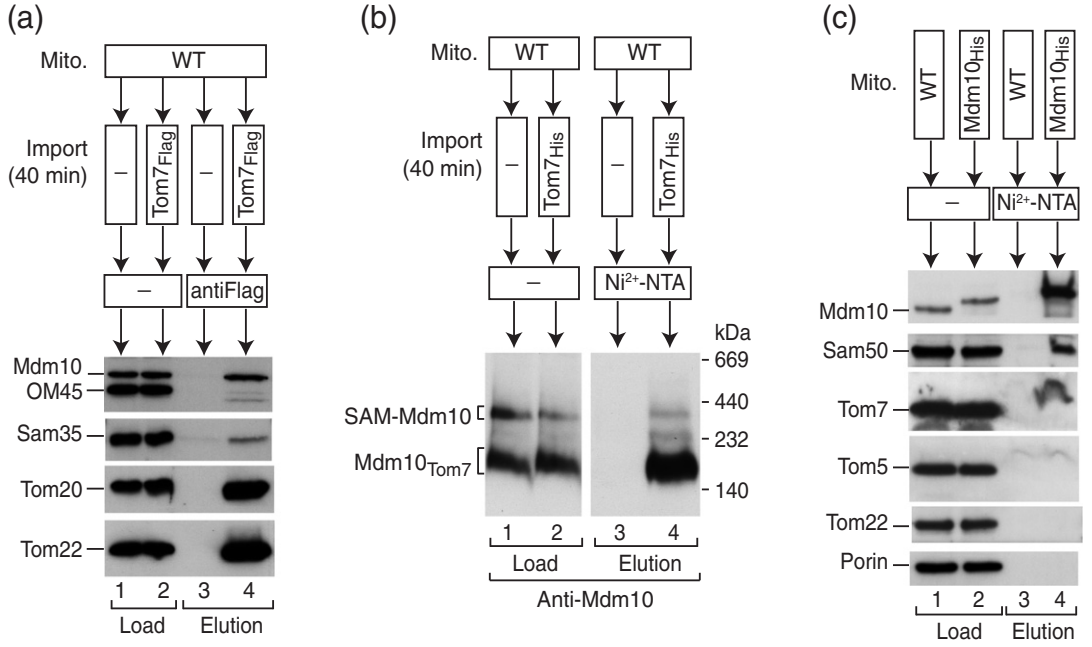

Fig. 3. Tom7 interacts with Mdm10. (a) Chemical amounts of $Tom7_{Flag}$ were imported into isolated wild-type (WT) mitochondria. Subsequently, mitochondria were lysed with digitonin and incubated with anti-Flag-coated Sepharose beads. Bound proteins were eluted and then analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. Load, 4%; elution, 100%. (b) Chemical amounts of $Tom7_{His}$ were imported into isolated wild-type mitochondria. Subsequently, mitochondria were lysed with digitonin and incubated with Ni–NTA column material. Bound proteins were eluted and then analyzed by blue native electrophoresis and immunodecoration with the indicated antibodies. Load, 5%; elution, 100%. (c) $MDM10_{His}$ and wild-type mitochondria were lysed with digitonin and incubated with Ni–NTA column material. Bound proteins were eluted and then analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. Load, 2%; elution, 100%.

complexes (SAM*) migrate at ~350 kDa (Fig. 2, lane 1).[47,49] The SAM–Mdm10 complex represents one of the large SAM forms (Fig. 2, lane 5) (Mdm10 additionally migrates in a lower molecular mass form of ~150 kDa).[47,49,50] Chemical amounts of Tom7 induced dissociation of the SAM–Mdm10 complex (Fig. 2, lanes 4 and 8). In contrast, Tom5 and Tom6 did not cause dissociation of the SAM–Mdm10 complex (Fig. 2, lanes 2, 3, 6, and 7). Neither the SAM_{core} complex nor the TOM complex was affected upon import of chemical amounts of the small Tom proteins (Fig. 2). We conclude that chemical amounts of Tom7 selectively destabilize the SAM–Mdm10 complex.

Together with the increased formation of the SAM–Mdm10 complex in mutant mitochondria lacking Tom7,[50] this finding raised the possibility that Tom7 may directly interact with Mdm10 and favor the generation of the SAM-free form of Mdm10. To address this hypothesis, we imported chemical amounts of Tom7 that carried an N-terminal Flag-tag into isolated mitochondria. The mitochondria were lysed with digitonin, and the imported Tom7 was purified via antibodies directed against the Flag-tag (Fig. 3a). Tom20 and Tom22 were co-purified with $Tom7_{Flag}$, indicating that $Tom7_{Flag}$ assembles into the TOM complex (Fig. 3a, lane 4). As control for the specificity of the purification, the abundant outer membrane protein OM45 was not present in the elution fraction. We found Mdm10 as an interaction partner in the elution fraction, whereas Sam35 was co-purified with low efficiency (Fig. 3a, lane 4). We asked which form of Mdm10 was preferentially associated with Tom7, the SAM–Mdm10 complex, or the SAM-free low molecular mass form. We imported Tom7 with a His-tag into mitochondria and purified the associated proteins by Ni–NTA affinity chromatography. Mdm10-containing protein complexes were analyzed by blue native electrophoresis (Fig. 3b). The low molecular mass, SAM-free form of Mdm10 was preferentially co-purified with tagged Tom7, whereas only small amounts of SAM–Mdm10 were co-purified (Fig. 3b, lane 4) (import of $Tom7_{His}$ also led to a partial destabilization of the SAM–Mdm10 complex; Fig. 3b, lane 2). To verify that Mdm10 interacts also with endogenous Tom7, we isolated mitochondria from a strain expressing His-tagged Mdm10. Mdm10 was purified by affinity chromatography. Tom7 was indeed present in the elution fraction (Fig. 3c, lane 4), whereas neither endogenous Tom22 nor Tom5 co-purified with Mdm10 (Fig. 3c, lane 4).[47,49] Thus, Tom7 interacts with Mdm10 in a TOM complex-independent manner. For comparison, Sam50 was co-purified with Mdm10 as it is a component of the SAM–Mdm10 complex (Fig. 3c, lane 4).[49,52,53]

We conclude that Tom7 preferentially binds to the SAM-free form of Mdm10. Tom7 thus shifts the equilibrium between SAM–Mdm10 and SAM-free Mdm10 towards the SAM-free form.

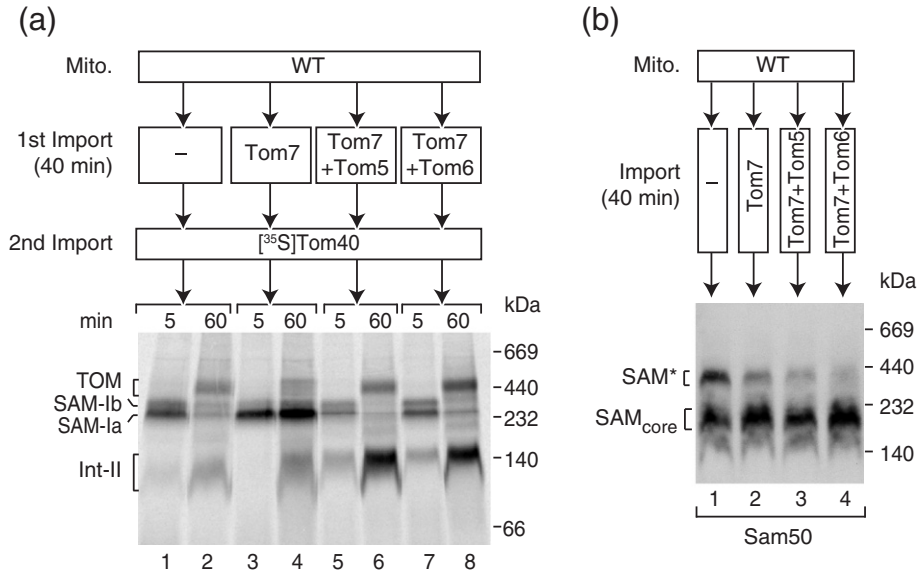

Fig. 4. Dual role of Tom7 in modulating biogenesis of the TOM complex. (a) Chemical amounts of Tom7 alone or in combination with Tom5 and Tom6 were imported into wild-type mitochondria. Mitochondria were reisolated and incubated with ^{35}S-labeled Tom40 for the indicated periods. Analysis was performed by blue native electrophoresis and digital autoradiography. (b) Chemical amounts of Tom7 alone or in combination with Tom5 and Tom6 were imported into wild-type mitochondria. Mitochondria were reisolated, lysed, and subjected to blue native electrophoresis. SAM complexes were detected by immunodecoration with the indicated antisera.

Differential effects of Tom7 on Tom40 biogenesis and SAM–Mdm10 complex

The results so far indicated that high levels of Tom7 delayed the biogenesis of Tom40 at the SAM complex and promoted dissociation of the SAM–Mdm10 complex. To determine if these effects were mechanistically coupled or not, we searched for conditions that differentially affected Tom40 biogenesis and the stability of the SAM–Mdm10 complex. We asked if Tom5 or Tom6 could counteract the effects of Tom7. We imported chemical amounts of Tom7 either alone or in parallel with Tom5 or Tom6 into mitochondria. The Tom7-

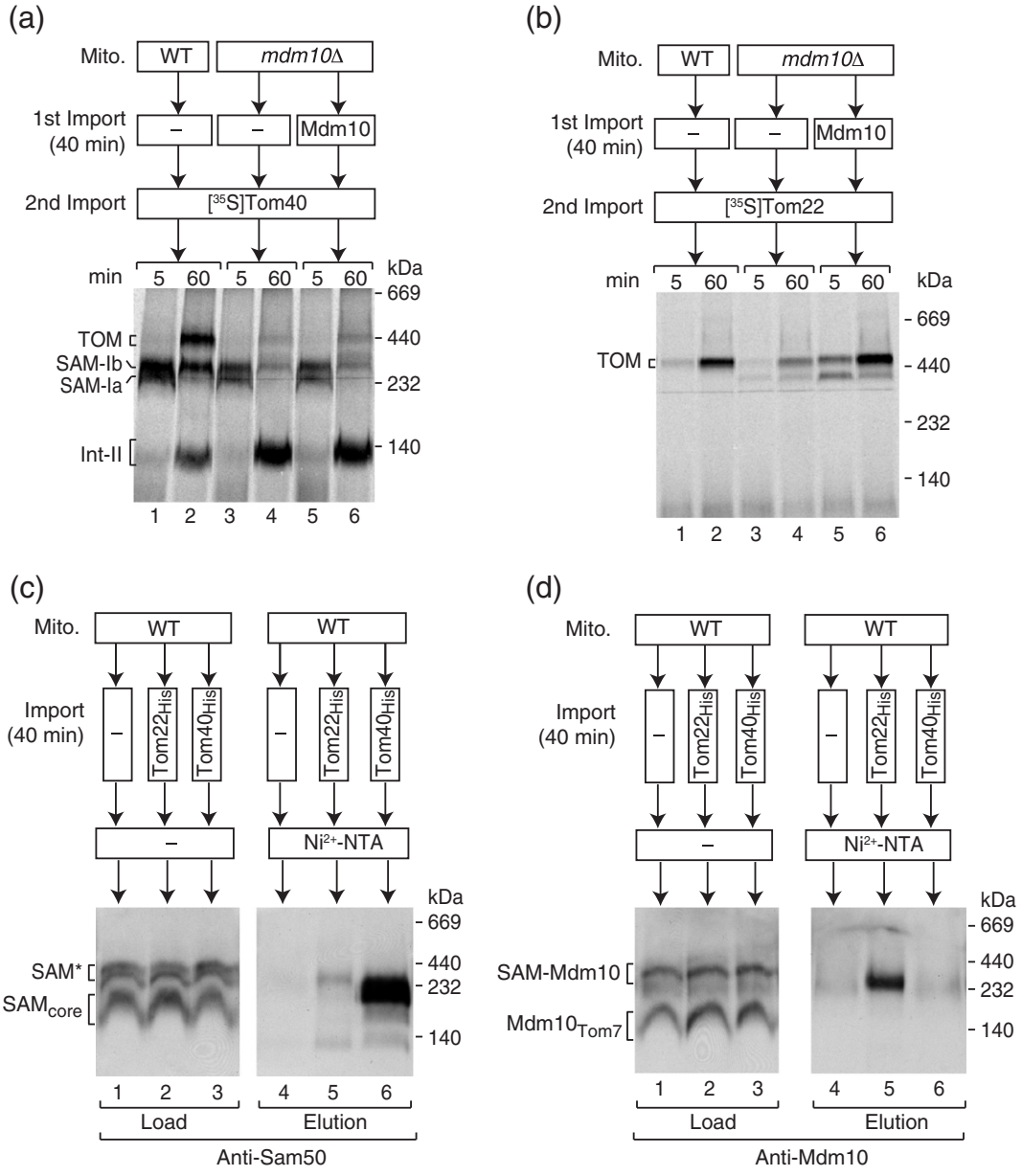

Fig. 5. Preferential function of the SAM–Mdm10 complex for biogenesis of Tom22. (a and b) Chemical amounts of Mdm10 were imported into wild-type and *mdm10*Δ mitochondria for 40 min. Mitochondria were reisolated and incubated with ^{35}S-labeled Tom40 or Tom22 for the indicated periods. Mitochondria were reisolated, lysed, and subjected to blue native electrophoresis and digital autoradiography. (c and d) Chemical amounts of $Tom22_{His}$ or $Tom40_{His}$ were imported into isolated wild-type mitochondria for 40 min. Subsequently, mitochondria were lysed and incubated with Ni–NTA column material. Bound proteins were eluted and subjected to blue native electrophoresis. Protein complexes were detected by immunodecoration with antibodies against Sam50 or Mdm10. Load, 3%; elution, 100%.

induced arrest of ^{35}S-labeled Tom40 at the SAM-Ia stage (Fig. 4a, lanes 3 and 4) was relieved by importing chemical amounts of Tom5 or Tom6, and the Tom40 precursor proceeded to later assembly steps, including the formation of SAM-Ib, intermediate II, and mature TOM complex (Fig. 4a, lanes 5–8). Next, we analyzed whether the import of high levels of Tom5 or Tom6 could also antagonize the Tom7-mediated dissociation of the SAM–Mdm10 complex. However, the presence of Tom5 or Tom6 did not interfere with the Tom7-induced dissociation of SAM–Mdm10 (Fig. 4b, lanes 2–4). We conclude that the early arrest of Tom40 assembly and the dissociation of the SAM–Mdm10 complex are two independent effects of the import of chemical amounts of Tom7.

SAM–Mdm10 complex stimulates Tom40 biogenesis by promoting assembly of Tom22

To characterize the role of Mdm10, we compared the assembly of the precursors of Tom40 and Tom22. ^{35}S-labeled Tom40 was imported into mitochondria lacking Mdm10. As reported, the formation of the mature TOM complex was strongly impaired, whereas both SAM stages, SAM-Ia and SAM-Ib, were formed and the Tom40 precursor accumulated at the intermediate II stage (Fig. 5a, lanes 3 and 4).[48–50] Surprisingly, when chemical amounts of Mdm10 were imported into mitochondria lacking Mdm10, the biogenesis of Tom40 was not restored (Fig. 5a, lane 6). In contrast, the impaired assembly of the precursor of Tom22 into the TOM complex in *mdm10*Δ mitochondria (Fig. 5b, lane 4)[49] was fully restored when chemical amounts of Mdm10 were imported (Fig. 5b, lane 6).

To determine which SAM forms stably interact with the precursors of Tom40 and Tom22, we imported chemical amounts of His-tagged Tom40 or Tom22. The mitochondria were lysed, and proteins associated with the tagged proteins were co-purified by affinity chromatography and analyzed by blue native electrophoresis. The SAM complexes were detected by immunodecoration for Sam50 and Mdm10 (Fig. 5c and d). $Tom40_{His}$ was efficiently bound to a Sam50-containing complex (Fig. 5c, lane 6) but not to the SAM–Mdm10 complex (Fig. 5d, lane 6). In contrast, $Tom22_{His}$ efficiently interacted with the SAM–Mdm10 complex (Fig. 5d, lane 5). These results indicate that the SAM–Mdm10 complex preferentially interacts with the precursor of Tom22 but not with the precursor of Tom40.

The preferential role of the SAM–Mdm10 complex for Tom22 raised the possibility that the assembly defect of Tom40 in *mdm10*Δ mitochondria may be caused by an insufficient supply of Tom22. We thus imported chemical amounts of Tom22 and Mdm10 into *mdm10*Δ mitochondria and studied the assembly pathway of ^{35}S-labeled Tom40. Under these conditions, the formation of the mature TOM complex was indeed fully restored (Fig. 6, lane 8). Even the pre-import of chemical amounts of Tom22 alone into *mdm10*Δ mitochondria considerably promoted TOM complex formation (Fig. 6, lane 6). Taken together, the SAM–Mdm10 complex functions in the biogenesis of Tom22, and the assembly defect of Tom40 in *mdm10*Δ mitochondria can be overcome by providing sufficient amounts of Tom22.

Tom7 affects the biogenesis of Tom22

It has been shown that the formation of the SAM–Mdm10 complex is increased in mitochondria lacking Tom7 (Fig. 7a, lane 2).[50] We thus asked if Tom7 affects the biogenesis of Tom22. We imported ^{35}S-labeled Tom22 into *tom7*Δ mitochondria and observed a more efficient assembly into the TOM complex than in wild-type mitochondria (Fig. 7a, lanes 3–8). The import of chemical amounts of Tom7 into wild-type mitochondria impaired the integration of Tom22 into the TOM complex (Fig. 7b, lane 8 compared to lane 2). To test whether this inhibitory effect was specific for Tom7, we loaded wild-type mitochondria with chemical amounts of Tom5 and Tom6. High levels of Tom5 did not influence the assembly of Tom22 (Fig. 7b, lane 4), whereas Tom6 stimulated the assembly of Tom22 into the TOM complex (Fig. 7b, lane 6). As shown in Fig. 4, co-import of chemical amounts of Tom5 or Tom6 along

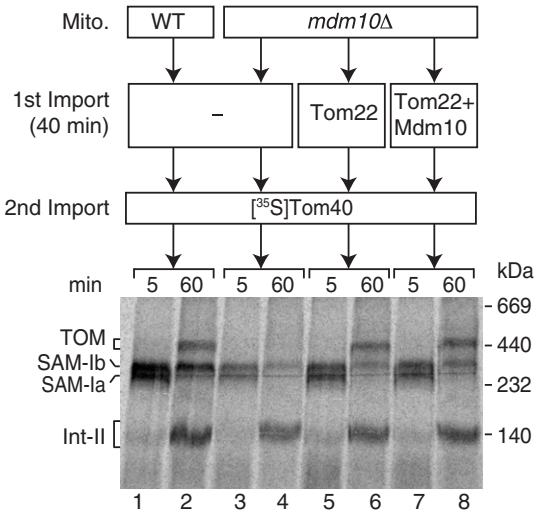

Fig. 6. High levels of Tom22 suppress the Tom40 assembly defect of *mdm10*Δ mitochondria. Chemical amounts of the indicated precursor proteins were imported into *mdm10*Δ mitochondria. Mitochondria were reisolated and incubated with ^{35}S-labeled Tom40 for 5 and 60 min at 25 °C. The mitochondria were analyzed by blue native electrophoresis and digital autoradiography.

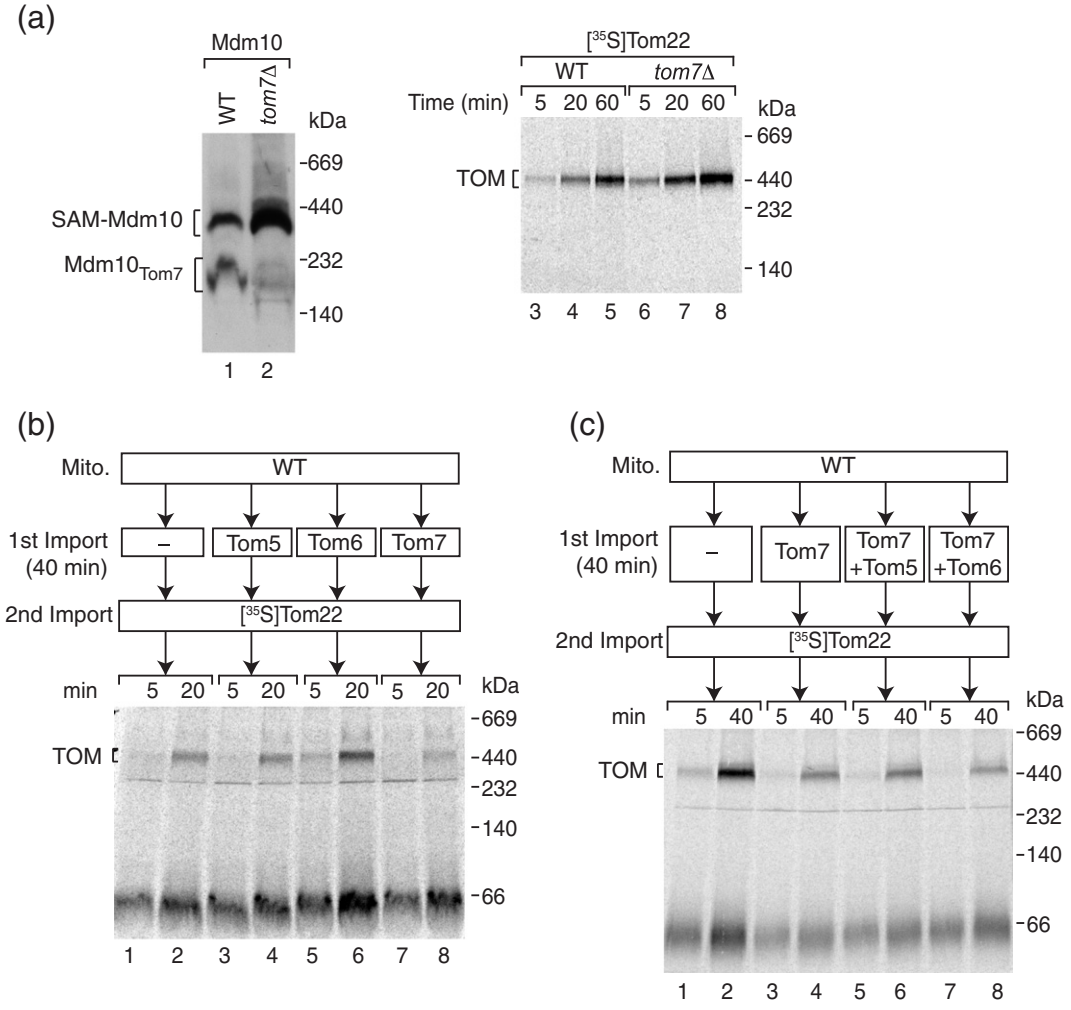

Fig. 7. Tom7 modulates Tom22 biogenesis via the SAM–Mdm10 complex. (a) Samples 1 and 2, protein complexes of wild-type and *tom7*Δ mitochondria were analyzed by blue native electrophoresis and detected with the indicated antiserum. Samples 3–8, ^{35}S-labeled Tom22 was imported into wild-type and *tom7*Δ mitochondria. Tom22-containing protein complexes were analyzed by blue native electrophoresis and digital autoradiography. (b) Chemical amounts of Tom5, Tom6, or Tom7 were imported into wild-type mitochondria for 40 min at 25 °C. After reisolation, ^{35}S-labeled Tom22 was imported for the indicated periods. Protein complexes were separated by blue native electrophoresis and analyzed by digital autoradiography. (c) Chemical amounts of Tom7 alone or in combination with Tom5 and Tom6 were imported into wild-type mitochondria. Mitochondria were reisolated and incubated with ^{35}S-labeled Tom22 for the indicated periods. Analysis was performed by blue native electrophoresis and autoradiography.

with Tom7 suppressed the inhibitory effect of Tom7 on the biogenesis of Tom40 but did not influence the Tom7-induced destabilization of the SAM–Mdm10 complex. To determine if the inhibitory effect of Tom7 on the biogenesis of Tom22 could be compensated for by Tom5 or Tom6, we imported chemical amounts of Tom7 alone or in combination with Tom5 or Tom6 into wild-type mitochondria and studied the assembly of ^{35}S-labeled Tom22. In contrast to the biogenesis of Tom40, the inhibitory effect of Tom7 on the assembly of Tom22 (Fig. 7c, lane 4) could not be overcome by the co-import of Tom5 or Tom6 (Fig. 7c, lanes 6 and 8). We conclude that Tom7 affects the biogenesis of Tom40 and Tom22 at different stages of assembly of the TOM complex.

Conclusions

We report that Tom7 modulates the biogenesis of the TOM complex at two distinct stages. (i) Tom7 delays the assembly of Tom40 at the SAM_{core} complex. The SAM_{core} complex binds the precursor of Tom40 and forms a platform for the association with Tom5 and Tom6.[47,48] Tom7 functions in an antagonistic manner to Tom5 and Tom6 at this early

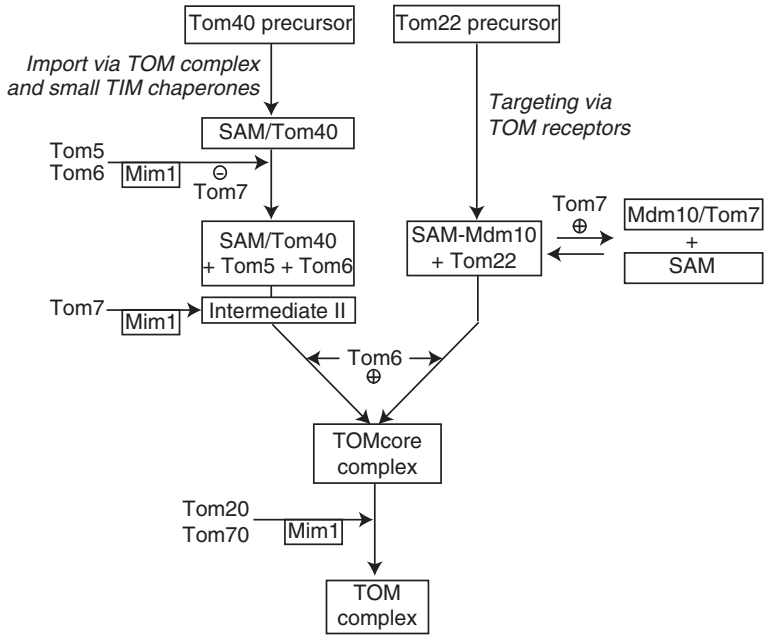

Fig. 8. Role of SAM complexes in the assembly of the TOM complex of the mitochondrial outer membrane (hypothetical model). For details, see the text (Conclusions).

stage (Fig. 8). (ii) Tom7 binds to Mdm10 and traps it in a SAM-free form, thus decreasing the amount of the SAM–Mdm10 complex (Fig. 8). This second function of Tom7 is counteracted by neither Tom5 nor Tom6. Our results provide strong support for a primary role of the SAM–Mdm10 complex in the biogenesis of Tom22,[45,47,49] whereas no evidence for a direct interaction of the SAM–Mdm10 complex with the precursor of Tom40 was obtained. Since the association of Tom22 with Tom40 is a crucial late step in the formation of the mature TOM complex,[17,19,22,49,57] the defect of the Tom22 pathway in *mdm10*Δ mitochondria leads to an impaired integration of Tom40 into the TOM complex. Tom7 delays the assembly of Tom22 by trapping SAM-free Mdm10 and thus reducing the SAM–Mdm10 levels. We conclude that Tom7 is not only a structural subunit of the TOM complex but also functions outside of the mature TOM complex as a regulatory factor at the SAM_{core} complex and as a binding partner of Mdm10.

Two models have been proposed for the function of Mdm10 at the SAM complex. Mdm10 may be involved in an early step of Tom40 biogenesis by mediating the release of the Tom40 precursor from the SAM complex, or the SAM–Mdm10 complex functions in late steps of maturation of the TOM complex that involves the association of Tom22 with Tom40.[45,47,49,53] Several lines of evidence favor the second model. First, release of the Tom40 precursor from the SAM complex and formation of the assembly intermediate II are not impaired in mitochondria lacking Mdm10.[48–50] Second, the SAM–Mdm10 complex binds the precursor of Tom22 and plays a primary role in its biogenesis. Third, chemical amounts of Tom22 imported into *mdm10*Δ mitochondria suppress the defect in Tom40 assembly. We conclude that the SAM–Mdm10 complex functions in the biogenesis of Tom22 and thus provides Tom22 for association with Tom40 at a late stage of TOM complex assembly.

Based on the findings reported here and in previous studies,[30–32,44–50,57] the following steps in the assembly of the TOM complex are proposed (Fig. 8). The precursor of Tom40 is initially imported via the TOM complex and the small TIM chaperones of the intermembrane space. The Tom40 precursor then binds to the SAM_{core} complex. Tom5 and Tom6 are inserted into the outer membrane in a Mim1-dependent manner and associate with the SAM-bound precursor of Tom40.[46–48] The module consisting of Tom40 and small Tom proteins can dissociate from the SAM complex to form intermediate II. Tom7 may associate with the SAM-free intermediate II.[57] The precursor of Tom22 is targeted to mitochondria via TOM receptors and binds to the SAM–Mdm10 complex, which is required for membrane insertion of the precursor and its assembly with intermediate II.[45,47,49] The MDM/ERMES complex may also be involved in the assembly pathway of the TOM complex.[51,52,56] Tom6 promotes late steps of TOM assembly, probably by stabilizing Tom40 molecules and supporting their association with Tom22.[19,22,23,44] Finally, membrane integration and assembly of the more loosely associated receptor proteins Tom20 and Tom70 depend on the presence of Mim1.[46,58,59]

Materials and Methods

Yeast strains and growth conditions

The *Saccharomyces cerevisiae* strains *mdm10*Δ, $MDM10_{His}$, and *tom7*Δ and the corresponding wild-type strains were described previously.[49,50] YPH499 was used as wild-type strain to isolate mitochondria for import experiments with chemical amounts. Yeast strains were grown on YPD, YPG, or YPS [1% (w/v) yeast extract, 2% (w/v) bactopeptone, 2% (w/v) glucose or 3% (w/v) glycerol or sucrose] at 19–24 °C.

In vitro import and blue native electrophoresis

Chemical amounts of outer membrane precursor proteins were synthesized using the wheat germ-based translation system RTS 100 Wheat Germ CECF Kit (5 Prime). PCR products generated by the RTS wheat germ linear template generation set (5 Prime) were used as template for the coupled transcription/translation reaction. Synthesis was performed in a 50-μl volume for 24 h at 25 °C under constant shaking. The efficiency of translation was controlled by Western blotting and immunodecoration. Mitochondria were isolated and stored according to published procedures.[60]The standard import of chemical amounts of precursor proteins was performed with 50 μg of mitochondria (protein amount) in import buffer [3% (w/v) fatty-acid-free bovine serum albumin, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 10 mM Mops/KOH (pH 7.2), 2 mM NADH, 4 mM ATP, 2.5 mM methionine, 5 mM creatine phosphate, 100 μg/ml creatine kinase] for 10–40 min at 25 °C. Per import reaction, 2–12% (v/v) wheat germ lysate was used. The import was stopped by transfer on ice. Subsequently, mitochondria were reisolated, washed with SEM buffer [250 mM sucrose, 1 mM EDTA (ethylenediaminetetraacetic acid), 10 mM Mops/KOH (pH 7.2)], and either solubilised with 1% digitonin for protein complex studies or used for the import of ^{35}S-labeled precursor proteins. Coupled and noncoupled *in vitro* translation systems (Promega) were used for the synthesis of ^{35}S-labeled precursor proteins in rabbit reticulocyte lysates. ^{35}S-labeled precursors were imported into isolated mitochondria at 25 °C as previously described.[60] The reaction was then transferred on ice. After washing with SEM buffer, mitochondria were solubilised with 1% digitonin in lysis buffer [20 mM Tris–HCl (pH 7.4), 50 mM NaCl, 0–10 mM imidazole, 0.1 mM EDTA, 10% (v/v) glycerol] and subjected to blue native electrophoresis.[60] After import of ^{35}S labeled precursor proteins, complexes and intermediates were visualized by digital autoradiography. Two-step import assays were essentially performed as described.[48] In brief, chemical amounts of precursor proteins were imported.[60] The import reaction was transferred on ice, and mitochondria were reisolated and washed. Subsequently, mitochondria were resuspended in a second import reaction and ^{35}S-labeled precursor proteins were added. Import reactions and subsequent analysis on blue native electrophoresis were performed as described earlier.

Pull-down of Flag- or His-tagged precursor proteins

Chemical amounts of N-terminally His-tagged Tom7, Tom22, and Tom40 and N-terminally Flag-tagged Tom7 were synthesized in the wheat germ system (5 Prime). The tagged proteins were imported for 40 min under standard conditions into isolated wild-type mitochondria. Subsequently, mitochondria were lysed with 1% digitonin in lysis buffer and incubated with Ni–NTA (Qiagen) or anti-Flag (Sigma) column material. Binding was performed for 1 h at 4 °C. The Ni–NTA column beads were washed with an excess amount of lysis buffer containing 20 or 40 mM imidazole and 0.1% digitonin. Bound proteins were eluted with 250 mM imidazole in lysis buffer containing 0.1% digitonin. For blue native electrophoresis, proteins were eluted with 1% digitonin and 250 mM imidazole in lysis buffer. Anti-Flag column material was washed with an excess amount of lysis buffer containing 0.1% digitonin. Bound proteins were eluted with 0.1 M glycine (pH 2.5). The eluted proteins were immediately neutralized and analyzed by Tris–tricine SDS-PAGE. The purification of Mdm10-containing complexes using a $MDM10_{His}$ strain was performed as described for the imported His-tagged Tom precursors.

Miscellaneous

Western transfer onto polyvinylidene difluoride membranes and immunodecoration were performed according to standard conditions. Enhanced chemiluminescence (GE Healthcare) was used following the recommendations of the manufacturer's manual.

Acknowledgements

We thank Nicole Zufall for expert technical assistance. This work was supported by the Baden-Württemberg Stiftung, Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 746, Trinationales Graduiertenkolleg GRK 1478, Excellence Initiative of the German Federal & State Governments (EXC 294 BIOSS; GSC-4 Spemann Graduate School), Bundesministerium für Bildung und Forschung, Landesforschungspreis Baden-Württemberg, Gottfried Wilhelm Leibniz Program, and the Fonds der Chemischen Industrie.

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