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Tom22 is a multifunctional organizer of the mitochondrial preprotein translocase

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Abstract

Mitochondrial preproteins are imported by a multisubunit translocase of the outer membrane (Tom), including receptor proteins and a general import pore (GIP) [145, 149, 159, 168, 169]. The central receptor Tom22 binds preproteins through both its cytosolic domain and its intermembrane space domain [17, 30, 83, 133, 137] and is stably associated with the channel protein Tom40 [48, 80, 196]. We report the unexpected observation that a yeast strain can survive without Tom22 although it is strongly reduced in growth and mitochondrial protein import. Tom22 is a multifunctional protein that is crucial for the higher level organization of the Tom machinery. In the absence of Tom22, the translocase dissociates into core complexes, representing the basic import units, but lacking a tight control of channel gating. The single membrane anchor of Tom22 is required for a stable interaction between the core complexes, while its cytosolic domain serves as docking point for the peripheral receptors Tom20 and Tom70. Tom22 reveals a novel functional principle of a preprotein translocase, combining receptor functions with distinct organizing roles in a multidomain protein.

Introduction

Nearly all mitochondrial proteins are synthesized in the cytosol and must be transported into the organelle. The translocase of the outer mitochondrial membrane (Tom) contains receptors for the specific recognition of preproteins and a general import pore (GIP) that mediates the transfer of different classes of preproteins across the outer membrane [48, 113, 121]. Subsequently the import pathways of preproteins diverge. Preproteins with amino-terminal signal sequences (presequences) are directed to a translocase of the inner membrane (Tim) that drives the transport across the inner membrane in cooperation with the heat shock protein 70 (mtHsp70) of the matrix. Hydrophobic proteins of the inner membrane carrier family and other inner membrane proteins are directed to a special Tim machinery consisting of several small subunits in the intermembrane space and a membrane integration complex (carrier translocase). Mitochondrial protein import is essential for cell viability since numerous subunits of the inner membrane translocase are indispensable for viability of the yeast Saccharomyces cerevisiae on both fermentable and non-fermentable carbon sources. This includes three Tim proteins of the presequence import pathway, plus mitochondrial Hsp70 whose translocase function is essential, and five Tim proteins of the carrier import pathway.

Surprisingly, only two out of nine Tom proteins, Tom22 and Tom40, were found to be essential for cell viability although the inner membrane pathways, which consist nearly exclusively of essential components, receive all their preproteins via the Tom machinery. Removal of other Tom proteins leads to phenotypes of different severity, including synthetic effects/lethality of
double deletions of several Tom protein encoding genes, indicating a complex network of functional cooperation of Tom proteins [50, 67, 74, 82, 138, 153]. The following functions have been assigned to yeast Tom proteins. At least three of them, Tom20, Tom22 and Tom70, function as receptors for preproteins. Presequence-containing preproteins are first recognized by Tom20, whereas carrier preproteins are preferentially targeted via Tom70. In addition, Tom70 cooperates with Tom37 [67], whereas Tom72, a homologue of Tom70, plays only a minor role in protein import. Both classes of preproteins are transferred to Tom22 as the second receptor level. With the help of Tom5, the preproteins are then inserted into the import channel formed by Tom40. The intermembrane space domain of Tom22 provides a trans binding site for preproteins. A complex of 400K was identified in yeast mitochondria, containing stably associated Tom40, Tom22 and three small Tom proteins (Tom7, Tom6 and Tom5), whereas Tom20, Tom70 and other Tom proteins are more loosely associated with this GIP complex [48]. Tom6 supports assembly of Tom22 with Tom40, while Tom7 favours a dissociation of the translocase.

While Tom22 and Tom40 are thus recognized as the essential core of the outer membrane translocase, little is known of how the multisubunit Tom machinery is coordinated, how the interaction of Tom subcomplexes and transfer of preproteins can proceed in an organized manner [168]. Here we report the astonishing observation that a yeast strain can survive without Tom22, providing the unique opportunity for defining the essential characteristics of the outer membrane translocase and identifying Tom22 as a multifunctional component in preprotein targeting and organization of the Tom machinery.

Materials and Methods

Generation and analysis of TOM22-deficient yeast
A BamHI/SalI fragment containing the TOM22 ORF was ligated into the high copy vector pFL44 with the MET25-promoter and the CYC1-terminator, yielding the plasmid BG3036 (URA3, TOM22). This plasmid was introduced into the diploid strain OL551 (his3-A200/his3-A200 leu2-Δ1/leu2-Δ1 ura3-52/ura3-52 trp1-Δ63/trp1-Δ63 TOM22/tom22::HIS3 rho+), which was subsequently sporulated and subjected to tetrad dissection. A haploid progeny (tom22::HIS3) containing BG3036 was then plated on medium with 5-fluoroorotic acid (5-FOA; [14]); colonies growing at 28°C were tested for the absence of the TOM22 gene and one of them was designated OL201 (his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 tom22::HIS3 rho0). The lack of mitochondrial DNA in OL201 cells was demonstrated by crossing the strain with a rho0 tester strain and by performing DAPI and HOECHST staining [105, 204]. The control haploid strain OL223 (his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 rho0) was generated by ethidium bromide mutagenesis of a TOM22 containing spore of an OL551 tetrad.

Analytical PCR on permeabilized yeast cells
The presence of either a wild type TOM22 gene, a disrupted tom22 gene or both in the yeast strains OL551, OL223 and OL201 was determined by performing a PCR reaction with the TOM22 specific oligonucleotides 5'-gcggcatcttcagagaacc-3' and 5'-catctttttcactgtgga-3'. These primers anneal in the extreme flanks of the coding region of the TOM22 gene, which remain present after disruption of the
**Northern analysis**

Yeast cultures in 25 ml YPD (2% (w/v) glucose, 2% (w/v) bactopeptone, 1% (w/v) yeast extract) were grown at 28°C until an OD600 of about 2.5 was reached. Total RNA was isolated according to De Winde et al. [44]. 20 µg of each RNA sample was run on a 1.2% TBE agarose gel, transferred to a Hybond-N+ filter (Amersham) which was then incubated in hybridization buffer (50% formamide, 5x SSC, 5x Denhardt's, 50mM Na2HPO4 (pH 6.5), 100 µg/ml salmon sperm DNA) containing a PDA1 probe, at 42°C overnight. The blot was washed with 3x SSC/0.1% SDS, 2x SSC/0.1% SDS and 1x SSC/0.1% SDS at 42°C for 30 min. each. Following autoradiography on Hyperfilm (Amersham), the PDA1 probe was removed from the blot by washing in 0.1% SDS at 95°C for 1 hr. and the blot was subsequently hybridized with a TOM22 probe. Probes were DNA fragments which were radiolabeled by nick-translation in the presence of 32P-α-dATP (Amersham) and consisted of either a 1333 bp NcoI-HindIII fragment of the PDA1 gene from the plasmid pAZ6 [202] or a 600 bp BamHI-SaI fragment of the TOM22 gene from the plasmid BG3036. The PDA1 gene (encoding the E1α subunit of the pyruvate dehydrogenase complex from *S. cerevisiae*) is constitutively transcribed and was used as an internal control [202].

**Southern analysis**

Yeast cultures in 25 ml YPD were grown at 28°C until stationary phase was reached. Chromosomal DNA was isolated according to Sambrook et al. [163]. 5 µg of DNA was digested with EcoRI, EcoRV, BglII or PsiI. Fragments were separated on a 0.8% 1.0 agarose gel, transferred to a Hybond-N+ filter (Amersham) which was then incubated in hybridization buffer (6x SSC, 5x Denhardt's, 100 µg/ml salmon sperm DNA, 0.5% SDS) containing the TOM22 probe, at 65°C overnight. The filter was then washed with 3x SSC/0.1% SDS, 2x SSC/0.1% SDS and 1x SSC/0.1% SDS at 65°C for 30 min. each, followed by autoradiography on Hyperfilm (Amersham).
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diploid strain and transformants were plated on selective minimal medium. One of the transformants was sporulated and subjected to tetrad analysis. Spores with the \textit{tom40} deletion (geneticin-resistant) and the plasmid YEplac33::ADH1p-TOM40 (ura\textsuperscript{+}) were subjected to the same genetic screen as used to attempt to isolate the \textit{tom22A} strain OL201 (Figure 1a), in an attempt to obtain a viable \textit{tom40A} strain in a similar way.

\textbf{Tetrad analysis}

Diploid strains were sporulated at 28°C in liquid sporulation medium (1% (w/v) KAc, 0.1% (w/v) glucose, 0.25% (w/v) yeast extract). Tetrad analysis was performed with a Singer semi-automatic microscope and spores were germinated at 28°C on YPD plates. The distribution of auxotrophic markers of viable spores was tested by replica plating to minimal growth media supplemented with the appropriate requirements.

\textbf{In vitro import of preproteins into mitochondria and cross-linking}

\textit{35}S-labeled precursor proteins were synthesized in rabbit reticulocyte lysate [189]. Preproteins were imported into isolated yeast mitochondria in a BSA-containing buffer (3% (w/v) BSA, 250 mM sucrose, 5 mM MgCl\textsubscript{2}, 80 mM KCl, 10 mM MOPS/KOH, pH 7.2), in the presence of 2-4 mM ATP, 2-4 mM NADH at 25°C. An energy-regenerating system (10-20 mM phosphocreatine, 100-200 μg/ml phosphocreatine kinase) was added to allow efficient import into \textit{rho}\textsuperscript{0} mitochondria. After the import reaction, mitochondria were treated with 50 μg/ml proteinase K for 15 min. at 0°C [2, 82]. The mitochondria were reisolated, washed and proteins were separated by SDS-PAGE. In cases where the import was studied in the absence of surface receptors, mitochondria were pretreated with 20 μg/ml trypsin for 20 min. at 0°C. Dissipation of the membrane potential was accomplished by the addition of 1 μM valinomycin, 20 μM oligomycin and 8 μM antimycin A [2, 189]. For cross-linking, \textit{35}S-labeled AAC-DHFR was accumulated at the outer mitochondrial membranes by the addition of 20 μM methotrexate to the import reaction. Mitochondria were reisolated and subjected to cross-linking with 0.4 mM EGS prior to immunoprecipitation under stringent conditions [2].

\textbf{Blue-native gel electrophoresis and co-precipitation of Tom proteins}

Mitochondria were lysed in 50 μl ice-cold digitonin buffer (1% (w/v) digitonin, 20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10% (v/v) glycerol, 1 mM PMSF) prior to the addition of sample buffer (5% (w/v) Coomassie brilliant blue G-250, 100 mM Bis-Tris, pH 7.0, 500 mM 6-aminocaproic acid). Blue native PAGE was performed using a 6-16.5% polyacrylamide gradient gel at 10°C [48]. Following electrophoresis, the gel was blotted onto PVDF membranes, or gel-strips were subjected to a second-dimensional SDS-PAGE.

For co-precipitation of Tom proteins from mitochondria, isolated mitochondria (250 μg of protein) were lysed in 0.5% digitonin-containing buffer and incubated with protein A-Sepharose to which antibodies were covalently coupled [2, 3, 82]. After washing in digitonin-containing buffer, the bound material was eluted by the addition of electrophoresis sample buffer and proteins were separated by SDS-PAGE. The proteins were transferred onto nitrocellulose and immunodecorated with various antisera.

For co-precipitation of purified Tom proteins, the expressed and purified cytosolic domains of Tom20, Tom22 or Tom70 (100 pmol, each) [30] were pairwise incubated in 50 mM KCl, 10 mM MOPS-KOH, pH 7.2, 0.5% BSA for 15 min. at 25°C (200 μl final volume). Aggregated material was removed by centrifugation for 20 min. at 106,000 x g. The supernatant was incubated for 1 h. at 4°C with anti-Tom antibodies coupled to protein A-Sepharose. After two washes with 50 mM KCl, 10 mM MOPS-KOH, pH 7.2, the precipitate was analyzed by SDS-PAGE, immunodecoration and Fluoro-imaging (Fuji).

\textbf{Electrophysiological measurements}

Planar lipid bilayers were made by using the painting technique [80, 81]. After formation of a stable bilayer (\textit{cis} chamber: 250 mM KCl, 10 mM CaCl\textsubscript{2}, 10 mM MOPS/Tris, pH 7.0; \textit{trans} chamber: 20 mM KCl, 10 mM MOPS/Tris, pH 7.0), outer membrane vesicles were added to the \textit{cis} chamber directly...
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Results

Viability of yeast in the absence of Tom22
Only two Tom proteins, Tom22 and Tom40, were reported to be essential for cell viability [5, 83, 122]. This was determined by a standard assay, i.e. disruption of one chromosomal copy of a gene in a diploid strain of the yeast Saccharomyces cerevisiae, followed by sporulation, dissection of tetrads and analyzing if the resulting haploid cells lacking the gene can grow [69]. Similarly, a sheltered disruption of Tom22 in Neurospora crassa led to the conclusion that Tom22 is essential for viability [144]. In a second approach for S. cerevisiae, we asked if TOM22 could be deleted directly from haploid cells (strain OL223) by a one step gene disruption. We therefore tried to disrupt the TOM22 gene in the haploid strain OL223 (wild type, rho+). For this purpose, a DNA fragment harbouring the HIS3 coding region flanked by TOM22 specific regions was introduced into strain OL223 and his+ transformants were selected on minimal medium plates. As a control, we also disrupted one of both TOM22 chromosomal copies in the diploid strain YPH501 using the same method. his+ transformants were subjected to PCR analysis to verify the presence of a disrupted TOM22 gene. However, no viable cells lacking the TOM22 gene could be detected in strain OL223, whereas one of both TOM22 genes could be disrupted in the diploid strain. In a third approach, a diploid yeast strain (OL551) lacking the TOM22 coding region in one of its chromosomal copies received a plasmid containing the wild type TOM22 gene and the URA3 marker. After sporulation and tetrad dissection, haploid cells containing the chromosomal disruption of TOM22 were selected, all of which carried the TOM22-URA3 plasmid. By addition of 5-fluoroorotic acid (5-FOA; [14]) to the selective plates, we found cells (OL201) that had lost the URA3-TOM22 plasmid and apparently lacked TOM22 (Figure 1a). To exclude that only the URA3 marker of BG3036 was inactivated while the TOM22 gene was still intact in OL201, we performed analytical PCR with primers flanking the TOM22 gene, generating a 409 bp fragment for the wild type TOM22 gene and a 1101 bp fragment for the disrupted gene (TOM22 flanking regions are still present in tom22Δ). OL201 contained only the disrupted gene (Figure 1b, lane 3), control haploid strain OL223 contained the wild type TOM22 gene (Figure 1b, lane 2), and diploid strain OL551 contained both the wild type and the disrupted gene (Figure 1b, lane 1). The absence of the TOM22 coding region in OL201 was also confirmed by Southern analysis. Total chromosomal DNA isolated from strain OL201, control haploid strain OL223 and diploid strain OL551 was subjected to single enzyme digestions using four different restriction enzymes.
Figure 1. A yeast strain lacking TOM22. a, Schematic representation of the genetic screen used to select for the tom22Δ strain OL201. b, Analytical PCR on chromosomal DNA of permeabilized cells of strains OL551 (lane 1), OL223 (lane 2) and OL201 (lane 3) yielding fragments characteristic for the intact TOM22 gene (409 bp; WT, wild type) and the HIS3-disrupted TOM22 gene (1101 bp; tom22Δ). c, Southern analysis of chromosomal DNA of strains OL551 (lanes 1-4), OL223 (lanes 5-8) and OL201 (lanes 9-12) digested with the restriction enzymes EcoRI (RI), EcoRV (RV), BglII (B) and PstI (P). Fragment sizes (bp) indicate the presence of wild type TOM22 (normal font) or disrupted tom22Δ (italic font). d, Northern analysis of total RNA of strains OL223 (lane 1) and OL201 (lane 2) showing the RNA levels for the constitutively expressed PDA gene (upper panel) and the TOM22 gene (lower panel).
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Each digestion yielded the expected fragments specific for the wild type TOM22 gene in OL223 (Figure 1c, lanes 5-8), for the deleted tom22 gene in OL201 (Figure 1c, lanes 9-12), and for both in diploid strain OL551 (Figure 1c, lanes 1-4). Finally, the lack of the TOM22 gene was confirmed by Northern analysis. Total RNA was isolated from strains OL201 and OL223. A band representing TOM22 RNA is present in control strain OL223, while this is lacking in strain OL201 (Figure 1d, lower panel). We conclude that OL201 lacks the TOM22 gene and henceforth refer to it as ‘tom22Δ’.

The tom22Δ cells were unable to grow in medium with non-fermentable carbon sources. By crossing strain OL201 with a rho0 tester strain, we demonstrated that the tom22Δ cells had defects in mitochondrial DNA (not shown). By DAPI and HOECHST staining the tom22Δ cells were found to be devoid of mitochondrial DNA (not shown) and are thus classified as rho0. Therefore the control haploid strain OL223 was derived from a TOM22 containing spore of sporulated OL551 and converted to a rho0 state by ethidium bromide mutagenesis (referred to as wild type with regard to TOM22) (Figure 1a). On fermentable carbon sources growth of tom22Δ cells was about four-fold slower than that of OL223 cells.

The frequency of spontaneous loss of the URA3 plasmid BG3036 from a control strain was about 1:10 while the frequency for obtaining OL201 was about 1:50. This indicates that about 20% of the cells could survive the loss of TOM22, which means that the frequency is too high for a genetic explanation of the phenomenon. Moreover, a back-cross of OL201 with the wild type haploid OL223 also did not provide evidence for a rapid generation of extragenic suppressor mutations to explain the viability of tom22Δ cells; the analysis of more than 90 tetrads revealed a 2:2 segregation of viability and all viable spores contained the wild type TOM22 gene (Figure 2). In case of the presence of an extragenic suppressor mutation we would expect 4, 2 and 3 viable spores to occur in a ratio of 1:1:4, which was clearly not the case.

![Figure 2](image)

This suggests that tom22Δ spores cannot germinate productively. The strong growth defects of tom22Δ cells provide a likely explanation why the cells did not survive the stress generated by two standard procedures for gene deletion, leading to the previous assumption that Tom22 is
essential for viability. Only the mild approach of spontaneous plasmid loss from growing cells allowed the recovery of a viable tom22Δ strain.

Mitochondria were isolated from tom22Δ cells and the corresponding wild type strain. Western analysis with a polyclonal anti-Tom22 serum demonstrated that the tom22Δ mitochondria completely lacked Tom22 (Figure 3, lane 2). No smaller fragments reacting with the antiserum were detected, even after extensive loading of the gel (Figure 3, lane 4).

The content of Tom20 in tom22Δ mitochondria was reduced to ~65% of the wild type level (Figure 3, lane 6). All other proteins tested were found in similar amounts in wild type and mutant mitochondria, including Tom70, Tom40 and Tom5 of the outer membrane (Figure 3, lane 6); Tim23, Tim22 and the ADP/ATP carrier (AAC) of the inner membrane; the β subunit of the F1-ATPase (F1β); and the matrix chaperonin Hsp60 (Figure 3, lane 8). We conclude that tom22Δ cells are viable, although only on medium with a fermentable carbon source and with a strong retardation of growth. The resulting mitochondria have a protein composition that is quite similar to control rho0 mitochondria and thus offer the unique opportunity to analyze the dependence of mitochondrial protein biogenesis on Tom22.

**Figure 3.** Analysis of the protein composition of tom22Δ mitochondria. Protein of isolated mitochondria (25 μg of protein) were separated by SDS-PAGE and analyzed by immunodecoration. Lanes 3 and 4 received 200 μg of mitochondrial protein.

**tom22Δ mitochondria are strongly impaired in protein import and interaction of peripheral receptors with the import pore**

To assay the protein import capacity of tom22Δ mitochondria, *in vitro* synthesized 35S-labeled preproteins were incubated with the isolated mitochondria (Figure 4a-c). Preproteins containing cleavable presequences were employed that were destined for the intermembrane space
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(cytochrome b<sub>2</sub>, not shown), the matrix side of the inner membrane (F<sub>1</sub>β) or the matrix (the α-subunit of the matrix processing peptidase (α-MPP), and a fusion protein between the presequence of F<sub>0</sub>-ATPase subunit 9 and dihydrofolate reductase (Su9-DHFR)). Non-cleavable precursors of AAC and the outer membrane protein porin were also used. *tom22A* mitochondria were able to import preproteins, but with a strongly reduced efficiency of about 5-15% compared to the wild type mitochondria (Figure 4a, b, compare lanes 5-7 to 1-3; Figure 4c, lanes 7-11 vs. 1-5). The import into or across the inner membrane of *tom22A* mitochondria was blocked by dissipation of the membrane potential (Figure 4a, b, lanes 8; Figure 4c, lane 12). By reexpression of Tom22 from a plasmid carrying the *TOM22* gene, protein import was largely restored (Figure 4b, lanes 9-11), demonstrating that the strong effects on protein import were Tom22-specific (the reduction of the Tom20 content would only slightly inhibit the import of cleavable precursors to about 80% of the wild type level [2]).

To monitor the association of preproteins with Tom proteins, we used an AAC-DHFR fusion protein. By addition of methotrexate, the carboxy-terminal DHFR is stabilized and does not cross the outer membrane [189] leading to the arrest of AAC-DHFR in the Tom machinery. Upon addition of the cross-linking reagent ethylene glycol bis-(succinimidylsuccinate) (EGS), Tom proteins in vicinity of the preprotein were identified by immunoprecipitation under stringent conditions. The efficiency of cross-linking of AAC-DHFR to the first receptor Tom70 was similar in wild type and *tom22A* mitochondria (Figure 4d, lanes 1 and 6), indicating that the lack of Tom22 did not impair the initial binding of the preprotein to the mitochondrial surface. However, crosslinking of AAC-DHFR to Tom40 was strongly reduced in *tom22A* mitochondria (Figure 4d, lane 7), indicating that transfer of the preprotein into the import pore was inhibited. Cross-linking to the second receptor Tom20 was also reduced (Figure 4d, lane 8), suggesting that the lack of Tom22 impairs the transfer of preprotein from Tom70 towards Tom20. Antibodies against the abundant porin did not precipitate cross-linking products of AAC-DHFR (Figure 4d, lanes 5 and 10), confirming the specificity of the approach.

Do the peripheral receptors Tom70 and Tom20 partially substitute for the lacking receptor function of Tom22? A pretreatment of mitochondria with trypsin removes the cytosolic domains of all receptors and protein import is reduced in wild type mitochondria to ~30% (bypass import) (Figure 4e) [2, 189]. With *tom22A* mitochondria, however, a trypsin pretreatment only slightly affected the residual import (Figure 4e). This result surprisingly suggests that without Tom22 preprotein import is largely independent of the typical receptor proteins, leaving the trypsin-resistant Tom5 as the only binding site for preproteins before insertion into the GIP [50]. A possible explanation would be that Tom22 is required for the docking of Tom20 and Tom70 to the GIP complex. The peripheral association of Tom20 and Tom70 with the GIP complex (Tom40) can be assayed by co-immunoprecipitation experiments under mild conditions [2, 3, 82].

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Figure 4. Inhibition of protein import into \( \text{tom22} \Delta \) mitochondria. a, Inhibition of preprotein import to different mitochondrial subcompartments. \(^{35}\)S-labeled preproteins were imported into isolated wild type or \( \text{tom22} \Delta \) mitochondria in the presence or absence of a membrane potential (\( \Delta \psi \)). After treatment with proteinase K, the reisolated mitochondria were subjected to SDS-PAGE and storage phosphor imaging technology. b, Restoration of protein import by reexpression of Tom22. Included are mitochondria isolated from a \( \text{tom22} \Delta \) strain reexpressing Tom22 (samples 9-12). The restoration of protein import was comparable if Tom22 was reexpressed from either a multi-copy vector (YEp13) or a single copy-vector (pRS415). p, i, m, precursor-, intermediate- and mature-sized forms of a protein, respectively. c, Import and assembly of the AAC are inhibited. Assembly of AAC to the dimeric form was assessed by blue-native PAGE. d, Cross-linking of AAC. AAC-DHFR was accumulated across the outer membrane in the presence of methotrexate. The reisolated mitochondria were subjected to cross-linking with EGS and analyzed by immunoprecipitation under stringent conditions. e, Bypass import into \( \text{tom22} \Delta \) mitochondria. Where indicated the mitochondria were pretreated with trypsin before the import of F1\( \beta \). Protein import into non-trypsined wild type mitochondria after 60 min. was set to 100% (control).

Antibodies against Tom40 co-precipitated Tom20 from wild type mitochondrial lysates, but not from \( \text{tom22} \Delta \) mitochondria (Figure 5a, lanes 3 and 4, column 10). Similarly, anti-Tom20 did not precipitate Tom40 from \( \text{tom22} \Delta \) mitochondria (Figure 5a, lane 6, column 11). Moreover, the precipitation of Tom40 with anti-Tom70 was blocked with \( \text{tom22} \Delta \) mitochondria (Figure 5a, lane 8, column 13). The specificity of the co-immunoprecipitation approach is underscored by the lack of precipitation of Tom40 or Tom20 by anti-Tom22 from \( \text{tom22} \Delta \) mitochondria (Figure 5a, lane 2). Upon reexpression of \( \text{TOM22} \) in the \( \text{tom22} \Delta \) strain, the association of Tom20 and
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Tom70 with Tom40 was restored to wild type levels (not shown). Thus the lack of Tom22 strongly reduces the interaction of the receptors Tom20 and Tom70 with the GIP.

Figure 5. Tom22 as docking point for Tom20 and Tom70.

a, The lack of Tom22 inhibits the association of Tom20 and Tom70 with Tom40 (GIP). Digitonin-lysed wild type and tom22Δ mitochondria were subjected to co-immunoprecipitation with antibodies directed against Tom22, Tom40, Tom20, or Tom70. The co-precipitated proteins were separated by SDS-PAGE and immunodecorated with antibodies directed against Tom40, Tom22, and Tom20. The amounts of precipitated Tom20 and Tom40 were quantified and the ratio of tom22Δ versus wild type is shown. b, Interaction of the cytosolic domain of Tom22 with the cytosolic domains of Tom20 and Tom70. The purified domains were pairwise mixed as indicated, followed by co-precipitations with antibodies directed against Tom20, Tom22, Tom70, or preimmune antibodies. The amount of direct precipitation of each domain with its genuine antibody was set to 1 (norm. units).

To determine if Tom22 directly interacts with Tom20 or Tom70, we assayed the interaction between the expressed and purified cytosolic domains of the receptors [30]. The cytosolic domains of Tom20 and Tom22 were mixed and subjected to co-immunoprecipitation. Tom20 was co-precipitated with anti-Tom22 (Figure 5b, column 1), whereas no co-precipitation was observed with preimmune serum (Figure 5b, column 2) or when Tom22 was omitted from the
incubation (not shown). Moreover, Tom22 was co-precipitated with anti-Tom20 (Figure 5b, column 5). Similarly, Tom22 and Tom70 could be co-precipitated (Figure 5b, columns 7 and 11). However, when Tom20 and Tom70 were mixed, only weak co-precipitation was observed (Figure 5b, columns 3 and 9), demonstrating the selectivity of association with Tom22. The previously reported transient interactions between Tom20 and Tom70 are apparently less stable and involve smaller fractions of the proteins [73], thereby escaping detection by our co-precipitation approach. We conclude that the purified cytosolic domain of Tom22 can associate with the cytosolic domains of Tom20 and Tom70.

Figure 6. The lack of Tom22 causes a dissociation of the 400K GIP complex. 

a, Dissociation to a 100K complex and restoration of the 400K complex by reexpression of Tom22. Mitochondria were isolated from wild type yeast (rho+), wild type (rho0), tom22Δ and a tom22Δ strain reexpressing Tom22. Samples were lysed in digitonin, subjected to blue-native PAGE and immunodecorated with anti-Tom40. b, Tom40, Tom7, Tom6 and Tom5 are present in the 100K complex. tom22Δ mitochondria were lysed in digitonin, separated by blue-native PAGE, followed by SDS-PAGE. Immunodecoration was performed with anti-Tom40 and anti-Tom5. 35S-labeled Tom7, Tom6 and Tom5 were imported into tom22Δ mitochondria and analyzed by 2D-PAGE and autoradiography. c, The membrane-spanning segment of Tom22 is required for stabilization of the 400K GIP complex.
Mitochondria were isolated from wild type yeast, the tom22-2 strain expressing Tom22 without its intermembrane space (IMS) domain, and the tom22Δ strain. Half of the samples were treated with proteinase K. The mitochondria were lysed in digitonin and analyzed by blue-native PAGE. Cyt., cytosolic side; OM, outer membrane. d, Accumulation of a preprotein in the 100K subcomplex of tom22Δ mitochondria. 35S-labeled AAC-DHFR was preincubated with methotrexate and imported into wild type and tom22Δ mitochondria. The mitochondria were lysed in digitonin and analyzed by blue-native PAGE and autoradiography (lanes 1-2). For lanes 3-4, digitonin-lysed mitochondria were analyzed by blue-native PAGE and immunodecoration for Tom40. e, Tom40 is present as a dimer in the 100K complex. 35S-labeled Tom7, Tom6 and Tom5 were imported into tom22Δ mitochondria. The mitochondria were lysed with Triton X-100 (samples 2-5) or digitonin (sample 1) and separated by blue-native PAGE. Analysis was by immunodecoration for Tom40 (samples 1 and 2) or autoradiography (samples 3-5). The asterisk indicates the positions of the small Toms close to the front of the gel.

The membrane anchor of Tom22 scaffolds the assembly of Tom core complexes to the GIP complex

In wild type mitochondria, Tom22, Tom40, and the three small proteins Tom5, Tom6 and Tom7 form the centre of the Tom machinery, the 400K GIP complex that remains stable on blue-native electrophoresis while the peripheral subunits Tom20 and Tom70 are mainly released [48, 50]. This complex was also observed in mitochondria from a rho0 strain (Figure 6a, lanes 1 and 2). With tom22Δ mitochondria, however, the 400K GIP complex, detected with anti-Tom40, completely dissociated to a complex of ~100K (Figure 6a, lane 3). Mitochondria isolated from the tom22Δ strain reexpressing Tom22 from a plasmid contained a large GIP complex (Figure 6a, lane 4), demonstrating that the dissociation of the GIP complex was selectively caused by the lack of Tom22. By two-dimensional electrophoresis of tom22Δ mitochondria and subsequent immunodecoration, Tom40 and Tom5 were quantitatively found in the 100K complex (Figure 6b, upper panel). The preproteins of Tom7 and Tom6, for which specific antibodies are not available, were synthesized and 35S-labeled in vitro and imported into the mitochondria. The bulk of Tom7 and Tom6 migrated at the 100K position (Figure 6b, lower panel) while a small amount of Tom6 was not assembled, as similarly observed with in vitro imported Tom5 (Figure 5b, lower panel) [48]. We conclude that the lack of Tom22 causes a dissociation of the GIP complex to a 100K complex that contains Tom40, Tom7, Tom6 and Tom5.

Which part of Tom22 is required for the integrity of the 400K GIP complex? A treatment of wild type mitochondria with protease removed the cytosolic domains of the Tom22 molecules in the GIP complex, causing a shift of the GIP complex to ~350K, but not to 100K (Figure 6c, lane 2) [48]. The GIP complex of tom22-2 mutant mitochondria, lacking the Tom22 intermembrane space domain [137], preferentially migrated as a large complex (Figure 6c, lane 3). When we removed both domains by treating tom22-2 mitochondria with protease, we still observed a large GIP complex and very little 100K complex (Figure 6c, lane 4). We conclude that neither the cytosolic domain nor the intermembrane space domain of Tom22 are required to maintain the stability of the bulk of GIP complexes. Only when the membrane anchor of Tom22 was
removed (tom22Δ mitochondria), the GIP complex quantitatively dissociated and the vast majority of Tom40 was found at the 100K position (Figure 6c, lane 5).

To determine if the 100K complex retained a functional protein import site, we arrested import of 35S-labeled AAC-DHFR across the outer membrane. The translocation intermediate accumulated at ~450K in wild type mitochondria (Figure 6d, lane 1) and at 150K in tom22Δ mitochondria (Figure 6d, lane 2). The size of the fusion protein is ~50K, indicating an accumulation in the 400K and 100K complexes, respectively. Thus the 100K subcomplex forms a functional import site. The wild type Tom complex contains about six molecules of Tom40 [48] and, as suggested by electron micrographic analysis in Neurospora crassa, up to three import channels [113], implying that a single import channel is formed by a dimer of Tom40. In order to test if the 100K subcomplex fulfilled this prediction, we used Triton X-100 to release the small Tom proteins from Tom40 (Figure 6e, lanes 3-5).[48]. Tom40, now representing the only component of the complex, migrated at 80K (Figure 6e, lane 2). Monomeric Tom40, obtained after lysis of mitochondria with SDS, migrates at 40-45K (not shown). We propose that a dimer of Tom40 present in the 100K subcomplex forms the basic unit of one import channel.

**Tom22-deficient import sites lose the tight control of channel gating**

To determine the electrophysiological characteristics of the translocase channel in tom22Δ outer membranes, we isolated outer membrane vesicles from wild type and tom22Δ mitochondria and analyzed them using the planar lipid bilayer technique [80]. A single type of cation channel was observed in both membranes (Figure 7a). We investigated the basic properties of the channel, i.e. conductance, selectivity, and sensitivity to the synthetic mitochondrial presequence of cytochrome oxidase subunit IV (CoxIV). The wild type channel revealed a reversal potential of \( E_{\text{rev}} = +30 \text{ mV} \) \( (P_{K^+}/P_{Cl^-} = 4.4:1) \), its slope conductance was \( \Lambda = 370 \pm 5 \text{ pS} \) for the main conductance and \( \Lambda_1 = 180 \pm 6 \text{ pS} \) for the most frequent subconductant level (Figure 7c, left panel). The channel of tom22Δ outer membranes revealed similar values: a reversal potential \( E_{\text{rev}} = +29 \text{ mV} \) with a slope conductance of \( \Lambda = 387 \pm 6 \text{ pS} \) and \( \Lambda_1 = 187 \pm 5 \text{ pS} \) for the main conductance and the most frequent subconductant level, respectively (Figure 7c, right panel).

As observed with purified Tom40 [80], the direct transitions between the conductance levels and the comparatively high frequency of the states suggest that they represent distinct conductance states of a single channel and not the coupled opening of two or more individual channels. In support of this view, the ratio between the frequency of the main conductance state and that of the subconductance state did not change significantly between wild type and tom22Δ outer membranes. The channels from both sources were highly sensitive to the presence of the CoxIV presequence peptide. The peptide induced a strong increase in the frequency of channel gating (flickering) of both the wild type channel and the tom22Δ channel (Figure 7b). This effect was reversible after removal of the peptide. Thus the basic properties of the channel are identical for
Tom22 is a multifunctional organizer of the mitochondrial preprotein translocase

wild type outer membranes, \textit{tom22}\(\Delta\) outer membranes and purified Tom40 [80]. However, a difference was observed for the open probability of the channel. While in wild type outer membranes the channel was mainly in the closed state (Figure 7a, left panel), the channel of \textit{tom22}\(\Delta\) outer membranes was mainly in the open state (Figure 7a, right panel), resembling the open state observed with purified Tom40 [80]. We conclude that Tom22 is required to negatively regulate the open probability of the Tom40 channel.

![Figure 7. Increased open probability of the import channel of \textit{tom22}\(\Delta\) mitochondria.](image)

**Discussion**

We report a new principle of organization of a multisubunit preprotein translocase. A multidomain protein, Tom22, not only functions as receptor and \textit{trans} binding site for preproteins, but it also organizes the interaction between the channel and receptor subcomplexes
at two levels. The following functions can be assigned to the three domains of Tom22. (i) The cytosolic domain plays a dual role, it specifically recognizes preproteins [30, 83, 133] and serves as docking point for the peripheral receptors Tom20 and Tom70. (ii) The intermembrane space domain provides a *trans* binding site for presequences [17, 137]. (iii) The single membrane anchor of Tom22 is crucial for the integrity of the GIP complex. In the absence of this membrane anchor, the GIP complex dissociates into small core complexes containing a dimer of Tom40 and the three small Toms. Such a 100K core complex probably contains a single channel that retains the basic channel properties but is already open in the absence of preproteins. In contrast, in the presence of Tom22, the wild type GIP complex contains tightly regulated channels (probably three channels [113]) that open when preproteins are present. Tom22 apparently represents a component of the machinery that controls the gate. Whereas Tom40 is the only strictly essential protein of the outer membrane translocase (cells lacking Tom40 were inviable with all methods of gene deletion applied) [5, 100]) and forms the basic import core, Tom22 is crucial for the organization of the core complexes into the large GIP complex, the interaction with the peripheral receptors and the efficient and coordinated transfer of preproteins. This multifunctional protein is thus the central organizer that converts a simple channel into a complex, dynamic and efficiently coordinated machinery for the recognition and translocation of preproteins.

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