

# Integration of tail-anchored proteins into the mitochondrial outer membrane does not require any known import components

Christian Kemper<sup>1</sup>, Shukry J. Habib<sup>1</sup>, Gertraud Engl<sup>2</sup>, Petra Heckmeyer<sup>1</sup>, Kai S. Dimmer<sup>2</sup> and Doron Rapaport<sup>2,\*</sup>

<sup>1</sup>Institut für Physiologische Chemie der Universität München, Butenandtstr. 5, 81377 Munich, Germany

<sup>2</sup>Interkultürliches Institut für Biochemie, Hoppe-Seyler-Str. 4, University of Tübingen, 72076 Tübingen, Germany

\*Author for correspondence (e-mail: doron.rapaport@uni-tuebingen.de)

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## Summary

Tail-anchored proteins form a distinct class of membrane proteins that are found in all intracellular membranes exposed to the cytosol. These proteins have a single membrane insertion sequence at their C-terminus and display a large N-terminal portion to the cytosol. Despite their importance for various cellular processes, the mechanisms by which these proteins are recognized at and inserted into their corresponding target membrane remained largely unclear. Here we address this issue and investigate the biogenesis of tail-anchored proteins residing in the mitochondrial outer membrane. To that goal we developed a highly specific assay to monitor the membrane insertion of the model tail-anchored protein Fis1. Using this assay, we show that in contrast to all other import pathways in yeast mitochondria, none of the import components at the outer membrane is involved in the insertion process of Fis1. Both the

steady-state levels of Fis1 and its *in vitro* insertion into isolated mitochondria were unaffected when mitochondria mutated in known import factors were analyzed. Fis1 was inserted into lipid vesicles, and importantly, elevated ergosterol contents in these vesicles inhibited this insertion. Collectively, these results suggest that Fis1 is inserted into mitochondria in a novel pathway where the unique lipid composition of the mitochondrial outer membrane contributes to the selectivity of the process. Thus, this work demonstrates a novel role for lipids in the biogenesis of mitochondrial protein.

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Key words: Mitochondria, Tail-anchored proteins, TOM complex

## Introduction

Tail-anchored (TA) proteins fulfil a variety of cellular functions and are found in all subcellular membranes facing the cytosol. These proteins have a single membrane insertion sequence at their C-terminus and display a large N-terminal portion to the cytosol (Borgese et al., 2003; Wattenberg and Lithgow, 2001). All TA proteins are imported post-translationally from the cytosol into their corresponding organelle by a mechanism that is not well resolved.

The mitochondrial outer membrane harbors a distinct set of TA proteins. Among them are: Fis1, a protein involved in fission of mitochondria (Mozdy et al., 2000), three small subunits (Tom5, Tom6, Tom7) of the translocase of the mitochondrial outer membrane (TOM complex) (Allen et al., 2002; Beilharz et al., 2003), regulators of apoptosis belonging to the Bcl-2 family (Cory and Adams, 2002), the mitochondrial form of cytochrome b5 (D'Arrigo et al., 1993), a synaptojanin-binding protein, OMP25 (Nemoto and De Camilli, 1999), and an alternatively spliced isoform of vesicles associated membrane protein, VAMP-1B (Isenmann et al., 1998). At least for some of these proteins it was shown that their tail-anchor domain is necessary and sufficient for targeting to mitochondria (Allen et al., 2002; Beilharz et al., 2003; Dembowski et al., 2001; Egan et al., 1999; Nguyen et al., 1993). The mitochondrial TA proteins do not share sequence conservation in their tail region and the mitochondrial-targeting information is rather encoded in structural features in this region. These features include

moderate hydrophobicity of the transmembrane segment (TMS), and a TMS that is not too long and contains positive charges at its flanking regions. It appears that the relative contribution of each of these structural features varies from protein to protein (Borgese et al., 2007; Rapaport, 2003).

Whereas the structural characteristics that allow the TA region to serve as a mitochondrial-targeting signal are quite well characterized, the mechanisms by which the TA region is recognized at the mitochondrial surface and inserted into the membrane are still largely unresolved. Conflicting reports exist regarding the requirements for surface receptors, external energy and cytosolic chaperones. On the one hand, the targeting of VAMP-1B was reported to rely on saturable surface receptors (Lan et al., 2000), and the import of Bcl-2 precursor into yeast mitochondria was proposed to involve the import receptor Tom20 (Motz et al., 2002). On the other hand, mitochondrial targeting of tail-anchored proteins in mammalian cells was proposed very recently to be independent of protease-sensitive proteins and of the TOM complex (Setoguchi et al., 2006).

Similarly unclear is how TA proteins are integrated into the membrane of the endoplasmic reticulum (ER). For some proteins, a mechanism that does not involve ER proteins was suggested (Brambillasca et al., 2006), whereas for others the SRP and the translocon at the ER were proposed to be involved (Abell et al., 2003; Abell et al., 2004). One potential reason for these conflicting

reports is the difficulty of finding a reliable *in vitro* assay to address this problem. For example, it is difficult to discriminate between nonspecific association of hydrophobic precursor proteins with the membrane and physiological membrane integration (Borgese et al., 2003).

To overcome this obstacle we developed a specific and reliable labeling assay to monitor the insertion of the model TA protein Fis1 into the mitochondrial outer membrane. We found that Fis1 is inserted via a novel import pathway where none of the known import components is involved. Remarkably, the defined lipid composition of the outer membrane is involved in this pathway by contributing to the specificity of the insertion into the correct intracellular compartment.

## Results

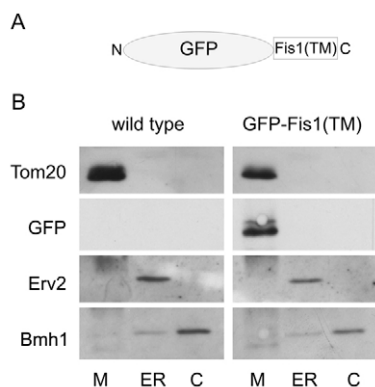
In the present study, we investigated the molecular mechanism by which TA proteins are inserted into the mitochondrial outer membrane. Previous studies concentrated on model tail-anchored proteins, which are subunits of the TOM complex (Dembowski et al., 2001; Horie et al., 2003). However, since these proteins may follow a special pathway, we decided to utilize Fis1 as a model protein. Fis1 is an outer-membrane protein that mediates fission of mitochondria, and its C-terminal transmembrane segment (TMS) was shown to be required for mitochondrial localization (Mozdy et al., 2000). Furthermore, human FIS1 lacking its N-terminal domain could still be targeted to mitochondria (Yoon et al., 2003). To demonstrate that the TMS is also sufficient for targeting of the protein to yeast mitochondria we constructed a fusion protein composed of GFP and the TMS of Fis1 (residues 129-155). Upon transformation of this construct into yeast cells and subcellular fractionation, the protein was found along with the marker protein Tom20 in the mitochondrial fraction (Fig. 1). Hence, the TMS of Fis1 is sufficient for targeting the protein, enabling its association with mitochondria.

A long-lasting problem in analyzing the integration of tail-anchored proteins into the mitochondrial outer membrane is the lack of a reliable assay to control for the correct insertion into the

membrane. Outer-membrane tail-anchored proteins expose only a few amino acid residues into the intermembrane space (IMS). Therefore, proteolytic cleavage of TA proteins with external protease results in the formation of protected fragments in the size range of 2-3 kDa, which are too small to be observed by SDS-PAGE. To overcome this problem we developed a labeling assay to study the insertion of Fis1 into the membrane. To that goal, the two cysteine residues in the cytosolic domain of native Fis1 were mutated to serine residues resulting in a Fis1 molecule that does not contain any cysteine residues in its sequence (Fis1-CS, Fig. 2A). Next, a serine residue (Ser147) in the putative TMS was replaced by a cysteine residue resulting in a Fis1 molecule with a single cysteine residue in the TMS (Fis1-TMC, Fig. 2A). This cysteine residue can be labeled by the membrane-impermeable sulfhydryl-reactive reagent IASD (4-acetamido-4'-[(iodoacetyl)amino]stilbene-2,2'-disulfonic acid). Such a labeling would occur only if the protein is not properly inserted into the membrane and hence the cysteine residue is not protected by the lipid bilayer. IASD has a molecular size of 624 Da and thus, its addition to Fis1 can be detected as a shift in the migration of the protein upon SDS-PAGE. This method was used successfully to study the topology of other membrane proteins, such as Bcl2,  $\alpha$ -hemolysin and the mitochondrial Mcr1 (Kim et al., 2004; Krishnasastri et al., 1994; Meineke et al., 2008). To demonstrate that this Fis1 variant is correctly targeted to mitochondria *in vivo* we used a functional complementation assay (Habib et al., 2003). Deletion of *FIS1* results in yeast cells that cannot undergo fission and therefore present mitochondria with altered morphology (Mozdy et al., 2000). Therefore, the ability of a variant to complement the morphology phenotype can be taken as a criterion for correct targeting and function in the outer membrane. Fis1-TMC was found to complement the morphology phenotype of *fis1* $\Delta$  demonstrating that this variant is functional in the outer membrane (Fig. 2B).

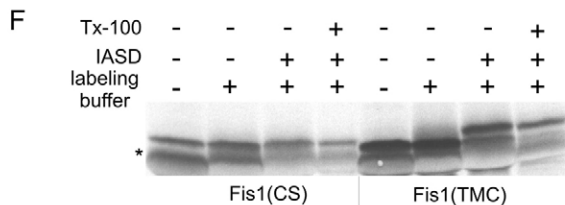
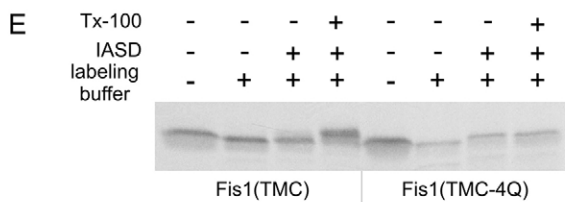
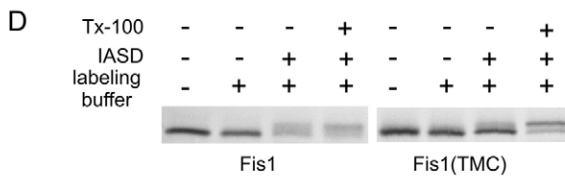
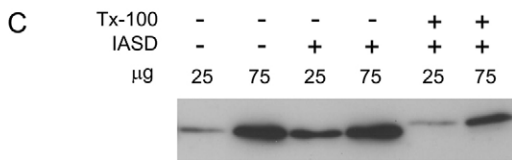
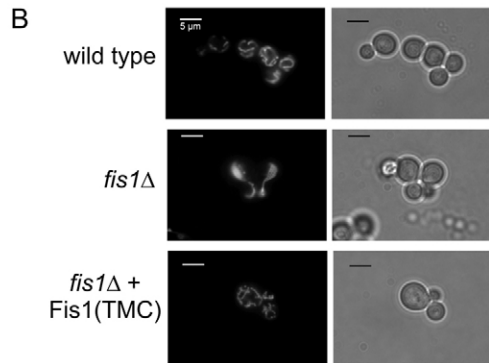
To check whether the anticipated membrane insertion can indeed be observed with this IASD-based assay, we isolated mitochondria from *fis1* $\Delta$  cells expressing plasmid-encoded Fis1-TMC and treated these mitochondria with IASD. As expected, because Cys147 is protected within the membrane, Fis1 molecules were not labeled with IASD. This protection disappeared upon solubilization of the organelle with the detergent Triton X-100 (Fig. 2C). Next, we incubated radiolabeled precursors of either native Fis1 or Fis1-TMC with isolated wild-type mitochondria, added IASD, and then treated the mitochondria with alkaline solution to remove soluble proteins. Both precursor proteins remained with the membrane fraction upon alkaline treatment of mitochondria. Furthermore, similarly to the endogenous Fis1-TMC, the majority of newly inserted Fis1-TMC molecules were protected from labeling unless detergent was added (Fig. 2D). Of note, a large fraction of wild-type Fis1 molecules, which harbor two cysteine residues in their cytosolic domain, were labeled even without detergent.

To validate the labeling system, we conducted control experiments using radiolabeled precursor proteins. First, we analyzed mitochondria in the IASD-labeling reaction using sucrose gradients (supplementary material Fig. S1). The IASD-unreacted radiolabeled Fis1 molecules were found in the same fractions as a marker outer-membrane protein, demonstrating that the inaccessibility of these molecules is not the outcome of aggregation. Next, we analyzed the labeling of a Fis1 variant where all positively charged residues in its C-terminal region were mutated to glutamine (Fis1-TMC-4Q, Fig. 2A). This variant cannot be inserted *in vivo* into the outer membrane and is nonfunctional (Habib et al., 2003).



**Fig. 1.** The TMD of Fis1 is sufficient for mitochondrial targeting. (A) Schematic representation of the GFP-Fis1(TM) protein. (B) Mitochondria (M) were purified via sucrose gradient from wild-type yeast cells or from cells transformed with a vector encoding GFP-Fis1(TM). The post mitochondrial fraction was further separated by differential centrifugation into ER fraction and cytosol (C). Proteins were subjected to SDS-PAGE and immunocytochemistry with antibodies against the GFP moiety, a control marker protein for the cytosol (Bmh1), a control ER marker protein (Erv2) and the mitochondrial outer-membrane protein Tom20.

**A** Fis1: M..C..C..TLKGVVVAGGVLGAVAVASFFLRNKRR  
 Fis1(CS): M..S..S..TLKGVVVAGGVLGAVAVASFFLRNKRR  
 Fis1(TMC): M..S..S..TLKGVVVAGGVLGAVAVACFFLRNKRR  
 Fis1(TMC-4Q):M..S..S..TLKGVVVAGGVLGAVAVACFFLQNNQQQ



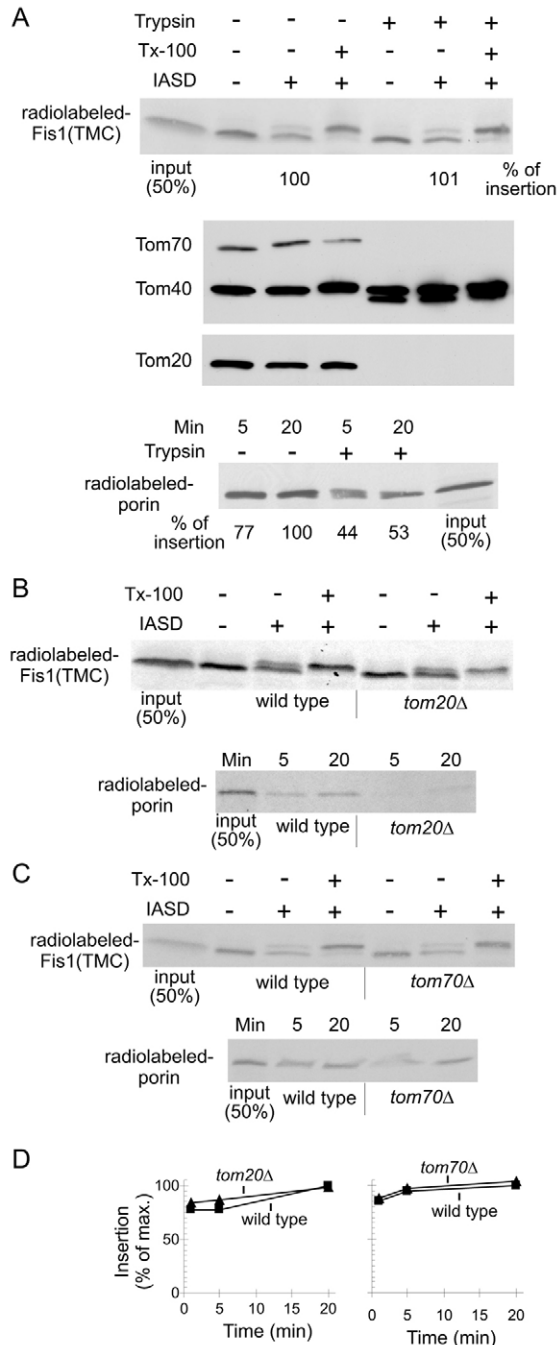
Accordingly, in contrast to Fis1-TMC, most of the Fis1-TMC-4Q was labeled with IASD, suggesting that its TMS was not inserted into the membrane (Fig. 2E). Since this variant has the same TMS as the Fis1-TMC variant, this experiment reveals that the protection from labeling by IASD is not simply due to nonspecific interaction of the hydrophobic TMS but results from correct insertion of the TMS into the membrane. Next, to demonstrate that the labeling of Fis1-TMC is indeed via its single cysteine residue we used a radiolabeled Fis1 variant with no cysteine residues (Fis1-CS, Fig. 2A). As is shown in Fig. 2F, no labeling was observed with this variant. Taken together, this labeling assay is specific and quantitative and can be used to study the mechanism of insertion of Fis1 into the mitochondrial outer membrane.

**Fig. 2.** Protection of Fis1-TMC from modification with IASD represents proper insertion into the outer membrane. (A) The sequences of the tail domains of native Fis1 and its variants. Only relevant residues in the cytosolic domain are indicated. The putative transmembrane segment is underlined. Cysteine and positively charged residues in the wild-type sequence and their replacement in the variant proteins are in bold type. (B) Fis1-TMC can complement the mitochondrial morphology phenotype of the *fis1Δ* strain. Cells of the indicated strains (containing mitochondria-targeted RFP) were analyzed by fluorescence (left) and phase-contrast (right) microscopy. (C) Endogenous Fis1-TMC is protected from modification with IASD. The indicated amounts of mitochondria isolated from a *fis1Δ* strain transformed with Fis1-TMC were incubated where indicated with IASD in the presence or absence of Triton X-100 (Tx-100). Mitochondrial proteins were analyzed by SDS-PAGE and immunocytochemistry with antibodies against Fis1. (D) Radiolabeled Fis1-TMC is protected from modification with IASD. Wild-type mitochondria were incubated with either radiolabeled Fis1 or radiolabeled Fis1-TMC for 30 minutes at 25°C. Mitochondria were re-isolated by centrifugation and resuspended in either import buffer or labeling buffer containing, where indicated, IASD and Tx-100. Mitochondrial proteins were analyzed by SDS-PAGE and autoradiography. (E) An insertion-deficient Fis1 variant is not protected from modification with IASD. Wild-type mitochondria were incubated with either radiolabeled Fis1-TMC or Fis1(TMC-4Q) for 30 minutes at 25°C. Further treatment was as described in D. (F) The modification of Fis1-TMC is cysteine specific. Radiolabeled precursors of Fis1(CS) and Fis1-TMC were analyzed directly by SDS-PAGE or were incubated for 20 minutes at 25°C in labeling buffer containing, where indicated, IASD and Tx-100. Further treatment was as described in D. A band representing hemoglobin, which is present at high amounts in the reticulocyte lysate is indicated with an asterisk.

Using the newly established *in vitro* assay, we examined whether the import receptors, Tom20 and Tom70 are required for the insertion pathway of tail-anchored proteins. Pretreatment of mitochondria with trypsin to remove any exposed parts of surface receptors did not result in any reduction of the insertion level of Fis1 (Fig. 3A). By contrast, the membrane integration of the  $\beta$ -barrel protein porin, which is known to require import receptors (Krimmer et al., 2001), was strongly reduced upon this treatment. Next, mitochondria isolated from strains lacking either Tom20 or Tom70 were incubated with radiolabeled Fis1-TMC and the amount of inserted precursor was analyzed. The absence of both receptors did not affect the insertion of the tail-anchored protein into the outer membrane (Fig. 3B-D). Of note, the rate of protein insertion was very fast. No significant differences in the insertion efficiencies were observed when the initial incubation at 0°C was performed for 1 minute or for 20 minutes (Fig. 3C). Similar results were obtained when other tail-anchored proteins, such as the small subunits of the TOM complex, were analyzed. The membrane insertion of precursors of Tom5, Tom6 and Tom7 was not affected by the absence of the import receptors (supplementary material Fig. S2A,B and data not shown). Collectively, the import receptors Tom20 and Tom70 seem to play only a minor role, if any, in the membrane insertion of tail-anchored proteins.

$\beta$ -barrel proteins are translocated through the import pore of the TOM complex before their insertion into the outer membrane (Paschen et al., 2005; Wiedemann et al., 2006). We next asked whether the import of Fis1 also requires the import pore formed by the TOM core complex. To this end, an excess of recombinant presequence-containing precursor protein that is known to use the import channel, pSu9(1-69)-DHFR, was added to the import reaction containing radiolabeled Fis1-TMC. This treatment did not influence the insertion of radiolabeled Fis1-TMC but, as expected, the import of the  $\beta$ -barrel protein porin was strongly inhibited under these conditions (Fig. 4A). Blocking the import pore also did not cause a reduction in the membrane insertion of the small Tom





subunits (supplementary material Fig. S2C and data not shown). Interestingly, such a treatment reduced the assembly of *Neurospora crassa* Tom6 and Tom7 into the TOM complex (Dembowski et al., 2001). Hence, it appears that clogging the import pore obstructs the assembly pathway of the small Tom proteins in a step downstream of the membrane integration. We further tested the insertion capacity of mitochondria lacking one of the small components of the TOM complex (Tom5, Tom6 or Tom7), or mutated in Tom40, the major component of the TOM complex (strains *tom40-2*, *tom40-3* and *tom40-4*). None of these mitochondria presented a reduced insertion ability (Fig. 4B,D). By contrast, the import of a presequence-containing precursor, pSu9-DHFR was reduced when mitochondria isolated from *tom6*Δ and *tom40-3* were

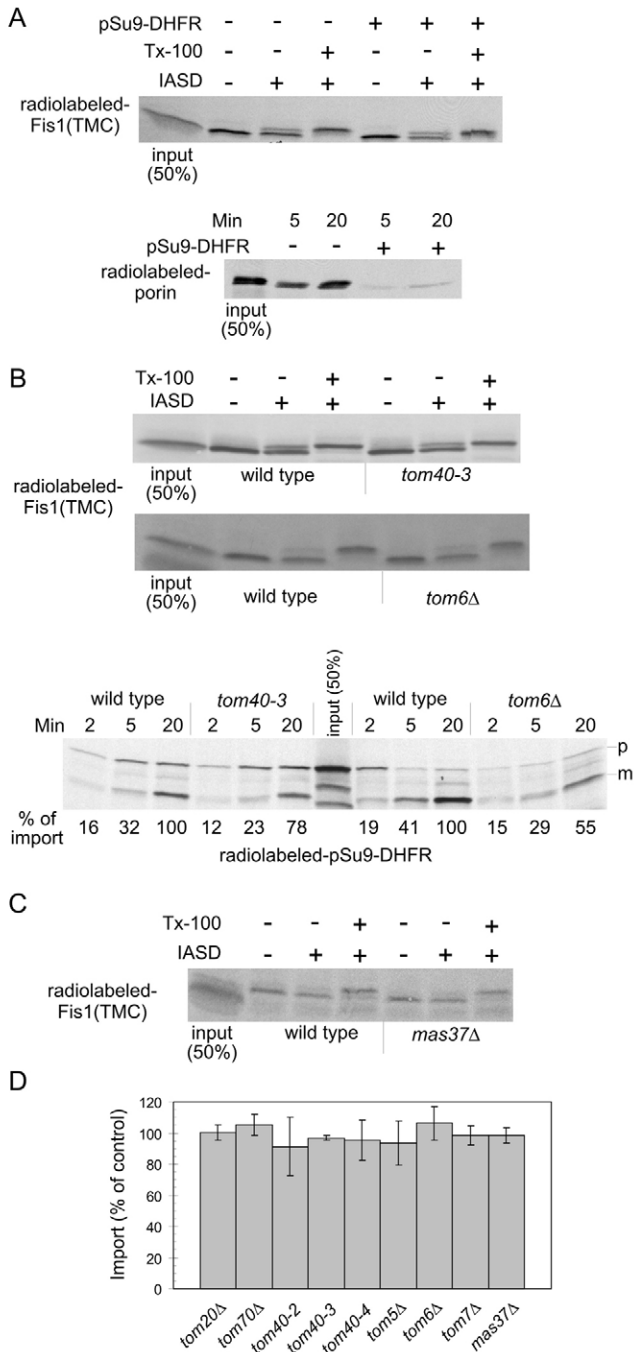
**Fig. 3.** The insertion of Fis1 does not require TOM import receptors.

(A) Radiolabeled precursor of Fis1-TMC was incubated with intact mitochondria or mitochondria pretreated with trypsin. Modification with IASD was as described above. Mitochondrial proteins were separated by SDS-PAGE, blotted to a membrane and then analyzed by autoradiography and immunocytochemistry. The antibodies used were directed against the receptor proteins Tom70 and Tom20 and the membrane-embedded Tom40, where a small fragment is cleaved off upon trypsin treatment. Lower panel: as a control, radiolabeled porin was incubated with the intact or trypsin-pretreated mitochondria for the indicated time periods. Mitochondria were treated with proteinase K (100 μg/ml) to degrade non-inserted protein and mitochondrial proteins were analyzed by SDS-PAGE and autoradiography. Insertion of Fis1 was calculated as the fraction of bound material (-IASD), which is protected in the presence of IASD (lower band in +IASD). The amount of precursor imported into intact mitochondria after 20 minutes was set to 100%. (B) Radiolabeled Fis1-TMC was incubated with mitochondria isolated from either the wild type or from cells lacking Tom20. Lower panel: as a control, radiolabeled porin was incubated with the same mitochondria and further treatment and analysis was as described in A. (C) Radiolabeled Fis1-TMC was incubated with mitochondria isolated from either the wild type or from cells lacking Tom70. Lower panel: as a control, radiolabeled porin was incubated with the same mitochondria and further treatment and analysis was as described in A. (D) Radiolabeled Fis1-TMC was incubated at 0°C for the indicated time points with either wild-type mitochondria or with mitochondria isolated from cells lacking either Tom20 or Tom70. Further treatment was as described in the legend to Fig. 2D. The bands were quantified and the intensity of the band corresponding to the unmodified protein was taken as a measure for protein insertion. The amount of protein inserted into mitochondria isolated from the corresponding wild-type strain after initial incubation for 20 minutes was set at 100%.

used (Fig. 4B, lower panel). The above mutations in Tom components also had no effect on the insertion of Fis1 when the kinetics of insertion at 0°C was analyzed (our unpublished results). Similarly, the membrane integration of the TOM small subunits was not affected when the mutated mitochondria were used (supplementary material Fig. S2D,E and data not shown). This TOM-independent behavior is in contrast to the insertion of the signal-anchored protein, Tom20, which was found to require Tom40 for obtaining its correct topology (Ahting et al., 2005).

The TOB-SAM complex, which is composed of Tob55, Tob38 and Mas37, mediates the membrane integration of β-barrel proteins after their passage through the TOM complex (Paschen et al., 2005; Pfanner et al., 2004). We asked whether this complex has an additional function as a mediator of the insertion of tail-anchored proteins. Neither deletion of Mas37 nor downregulation of the expression level of the essential proteins, Tob55 and Tob38 caused any reduction in the observed integration of Fis1-TMC or the small Tom subunits into the outer membrane (Fig. 4C,D, supplementary material Fig. S2F, and data not shown). Thus, TA proteins are inserted into the outer membrane in a process that is independent of the TOB complex. These results are in accordance with a recent report that Mas37 is required for the assembly of the small Tom proteins but not for their membrane insertion (Stojanovski et al., 2007). Of note, the steady-state levels of Fis1 in mitochondria mutated in either the TOM or TOB complex were similar to those in wild-type mitochondria (C.K., unpublished results). Taken together, none of the proteins that we examined appears to be essential for the insertion of tail-anchored proteins.

We asked whether Fis1-TMC could be inserted into lipid vesicles. We formed liposomes from a lipid mixture that mimics the published lipid composition of yeast mitochondrial outer membrane (De Kroon et al., 1999). Next, we tested the capacity of these liposomes to allow insertion of Fis1 compared with that of isolated mitochondria. The lipid content of the isolated mitochondria was estimated according to a previous publication (Gaigg et al., 1995), and similar

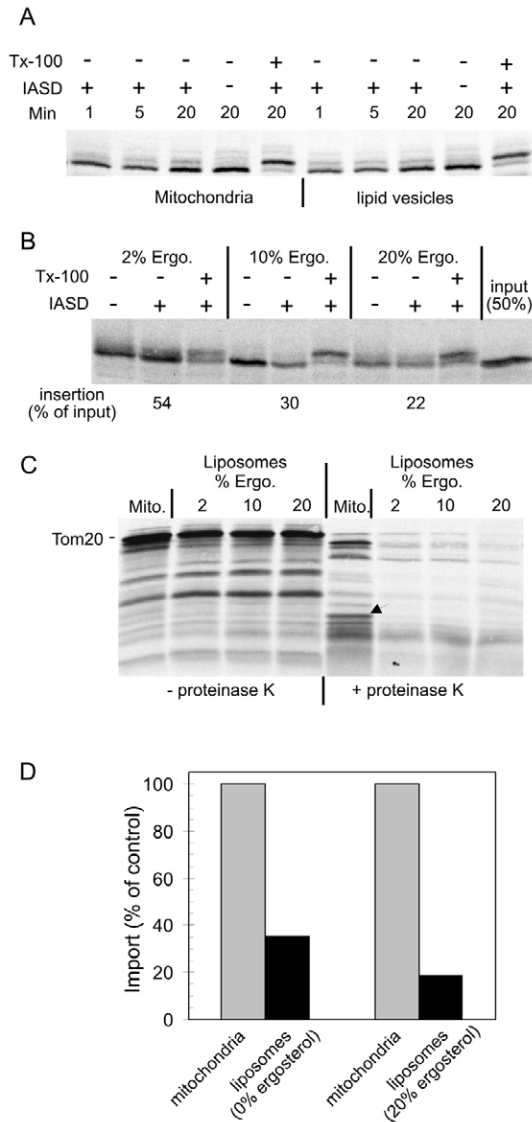


**Fig. 4.** Known import components in the outer membrane are not required for the insertion of Fis1. (A) Radiolabeled precursor of Fis1-TMC was incubated with isolated mitochondria in the presence or absence of excess recombinant matrix-destined precursor pSu9-DHFR. Further modification with IASD was as described in Fig. 2D. Lower panel: as a control, radiolabeled porin was incubated with isolated mitochondria in the presence or absence of excess pSu9-DHFR for the indicated time periods. Mitochondria were treated with proteinase K and mitochondrial proteins were analyzed by SDS-PAGE and autoradiography. (B) Radiolabeled Fis1-TMC was incubated with either wild-type mitochondria or with mitochondria isolated from cells harboring a temperature-sensitive allele of Tom40 (*tom40-3*) or lacking Tom6 (*tom6Δ*). Further treatment was as described in the legend to Fig. 2D. Lower panel, as a control, radiolabeled pSu9-DHFR was incubated with the indicated type of mitochondria for various time periods. The band corresponding to the mature form was quantified and the amount of precursor protein imported into control mitochondria after incubation for 20 minutes was set to 100%. Note that each mutant strain has different parental wild type strain. The precursor and mature forms of pSu9-DHFR are indicated with p and m, respectively. (C) Radiolabeled Fis1-TMC was incubated with either wild-type mitochondria or with mitochondria isolated from cells lacking Mas37 (*mas37Δ*). Further treatment was as described in Fig. 2D. (D) Experiments similar to that presented in Fig. 4B,C were performed with the indicated mutated strains and their corresponding wild-type strain. For each strain at least three independent experiments were performed. The bands corresponding to the inserted protein (unmodified protein in the presence of IASD, lower band in +IASD) were quantified. For each mutated strain the amount of protein inserted into the mutated mitochondria was compared with that inserted into mitochondria isolated from the corresponding wild-type strain, which was set to 100%. The error bars represent s.d.

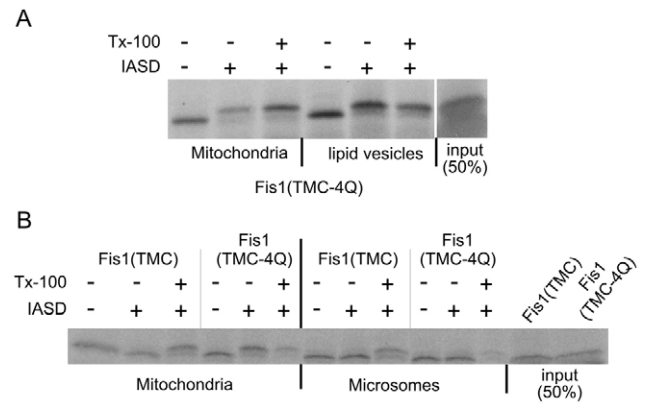
control, we tested by proteolytic assay the insertion of Tom20 into the lipid vesicles used in our assay. We observed before that the TOM complex is involved in the correct insertion of Tom20 into the outer membrane, and therefore Tom20 is inserted only in background levels into lipid vesicles (Ahting et al., 2005). As expected, Tom20 was attached to both lipid vesicles and mitochondria but was inserted only into the latter to acquire its correct topology (Fig. 5C). Thus, not every outer-membrane protein is inserted into lipid vesicles. Collectively, these experiments show that Fis1 is able to integrate into lipid vesicles that have a low ergosterol content.

The previous experiments were not performed under competitive conditions, and one can expect that the hydrophobic part of Fis1 will be inserted *in vitro* into any appropriate membrane with the correct lipid composition. Since the kinetics of insertion is too fast to be measured by our assay, we cannot compare the rate of insertion into lipid vesicles with that into mitochondria. Therefore, we asked to which membrane Fis1 would preferentially insert when both lipid vesicles and mitochondria are present in the same reaction mixture. These conditions better mimic the *in vivo* situation where TA proteins should avoid inappropriate insertion into the wrong compartment. To analyze the insertion into mitochondria or lipid vesicles separately, we used a differential centrifugation scheme to separate both components. To directly monitor the distribution of the lipid vesicles in this procedure we included 2 mol% PE-Fluorescein in the lipid vesicles and followed the fluorescence of the various fractions. Only 8-12% of the total fluorescence was observed with the mitochondrial fraction whereas ~90% was detected with the lipid vesicles (data not shown). Thus, we can exclude the possibility that the mitochondrial fraction was significantly contaminated with aggregated liposomes. Clearly a larger portion of Fis1 molecules was integrated into mitochondria compared with the lipid vesicles (Fig. 5D). The inclusion of ergosterol in the lipid vesicles reduced considerably their ability to compete with mitochondria in the insertion of Fis1 (Fig. 5D). These

concentrations of phospholipids were used in the samples with the lipid vesicles. As shown in Fig. 5A, the lipid vesicles had a similar insertion efficiency for TA proteins as observed in isolated mitochondria. From all subcellular membranes facing the cytosol, the outer membrane of mitochondria has the lowest ergosterol content namely, ergosterol/phospholipid ratio of 0.02/1 mol/mol (Schneider et al., 1999; Zinser et al., 1991). Hence, we reasoned that lower ergosterol levels can be part of the mechanism that secures the specific localization of mitochondrial tail-anchored proteins. To test this hypothesis, we prepared lipid vesicles with 2, 10 or 20 mol% of ergosterol and investigated their capacity to insert Fis1-TMC. Clearly, the inclusion of ergosterol in the lipid vesicles had an inhibitory effect on the insertion of Fis1-TMC (Fig. 5B). As a



**Fig. 5.** High ergosterol content reduces the efficiency of insertion of Fis1 into the membrane of lipid vesicles. (A) Radiolabeled precursor of Fis1-TMC was incubated for the indicated time periods with either mitochondria (50  $\mu$ g protein) or an equivalent amount of lipid vesicles (33  $\mu$ g lipids). Further treatment was as described in Fig. 2D. (B) Radiolabeled precursor of Fis1-TMC was incubated with lipid vesicles containing the indicated mol% of ergosterol. Further treatment was as described in Fig. 2D. The band that represents unlabeled Fis1 molecules in the presence of IASD (lower band in +IASD) was quantified by densitometry. Insertion was calculated as the intensity of this band in comparison to the amount of precursor protein added to the reaction. (C) Radiolabeled precursor of Tom20ext (Ahting et al., 2005) was incubated for 20 minutes at 25°C with either mitochondria or an equivalent amount of lipid vesicles containing the indicated mol% of ergosterol. After incubation, the samples were halved and in one half mitochondria or liposomes were pelleted and solubilized directly in sample buffer (-proteinase K). The other aliquots were treated with PK (500  $\mu$ g/ml) before solubilization in sample buffer (+proteinase K). The specific membrane-inserted proteolytic fragment (Ahting et al., 2005) is indicated with an arrowhead. (D) Radiolabeled precursor of Fis1-TMC was incubated with a mixture of mitochondria (50  $\mu$ g protein) and lipid vesicles (33  $\mu$ g). Modification with IASD was as described in Fig. 2D. Mitochondria were separated from lipid vesicles by differential centrifugation and proteins were analyzed by SDS-PAGE and autoradiography. The bands were quantified and the intensity of the band corresponding to the unmodified protein was taken as a measure for protein insertion. For each mixture, the amount of protein inserted into mitochondria was set to 100%.



**Fig. 6.** A charge variant of Fis1 is inserted into microsomes but not into the membrane of mitochondria or lipid vesicles. (A) Radiolabeled precursor of Fis1(TMC-4Q) was incubated for 20 minutes at 25°C with either mitochondria (50  $\mu$ g protein) or lipid vesicles (33  $\mu$ g lipids). Further treatment was as described in the legend to Fig. 2D. (B) Radiolabeled precursors of either Fis1-TMC or Fis1(TMC-4Q) were incubated for 20 minutes at 25°C with 50  $\mu$ g of either mitochondria or microsomes. Further treatment was as described in Fig. 2D.

results suggest that ergosterol inhibits the insertion of Fis1-TMC into lipid vesicles.

Next, we analyzed the membrane insertion of the Fis1 variant with charge replacements in its C-terminal region [Fis1(TMC-4Q), Fig. 2A]. In contrast to native Fis1, this variant could not be inserted into the membrane of lipid vesicles (Fig. 6A). To check the specificity of the *in vitro* system, we incubated Fis1(TMC) and its charge variant Fis1(TMC-4Q) with isolated microsomes. In agreement with its location *in vivo*, Fis1(TMC-4Q) was inserted into the membrane of microsomes (Fig. 6B) (Habib et al., 2003). Interestingly, Fis1(TMC), which was localized *in vivo* exclusively to mitochondria, was also inserted into microsomes (Fig. 6B). These results are in accordance with a previous report demonstrating that a cytochrome b5 variant that was targeted to mitochondria *in vivo* can efficiently insert into microsomes *in vitro* (Borgese et al., 2001).

## Discussion

A major question in the topogenesis of TA proteins is whether their delivery from the cytosol to the correct organelle membrane and the subsequent insertion into this membrane are assisted by cytosolic proteins and/or proteins in the target membrane. Conflicting results exist for TA proteins residing in the ER membrane (Borgese et al., 2003; High and Abell, 2004). For Syb2 the involvement of the Sec machinery was proposed (Abell et al., 2003), whereas a mechanism that does not require the assistance of other proteins was suggested for cytochrome b5 (Brambillasca et al., 2005). A possible solution for this confusion is the proposal that TA proteins can follow distinct pathways depending on the hydrophobicity of the TMS (High and Abell, 2004). Proteins with low hydrophobicity in this region can follow an unassisted pathway, whereas those with more hydrophobic TMS require additional proteins (Brambillasca et al., 2006). This idea is supported by the recent identification of a cytosolic protein, Asna1/TRC40, which interacts with the TMS of a subgroup of TA proteins and facilitates their targeting to the ER (Stefanovic and Hegde, 2007). Of note, TRC40 was not required for the insertion of proteins such as cytochrome b5, which can follow the unassisted pathway. Another example of the involvement of cytosolic proteins



is the finding that the membrane insertion of the ER TA protein Sec61 $\beta$  is facilitated by the chaperone Hsp70 and its co-chaperone Hsp40 (Abell et al., 2007). To date, a protein facilitating the biogenesis of mitochondrial TA proteins was not identified.

The vast majority of studies on TA proteins in mitochondria concentrate on subunits of the TOM complex as model proteins. Thus, it is not surprising that the correct topology and assembly of such TA proteins depends on a pre-existing TOM complex (Dembowski et al., 2001). In the current study, we chose to use Fis1 as a model protein because it is not a subunit of the TOM complex. Our results suggest that the TOM complex is not involved in the insertion of this TA protein. In a previous study, the import receptor Tom20 was reported to be involved in the insertion of another TA protein, Bcl-2 into yeast mitochondria (Motz et al., 2002). It is not clear whether this discrepancy is caused by the difference in the model protein and assay used or is due to the heterologous system used by Motz and colleagues. However, our results do agree with a recently published study where the insertion of various TA proteins into mammalian mitochondrial outer membranes was reported to be independent of TOM (Setoguchi et al., 2006). The current study does not only substantiate this previous report but rather utilizes the advantages of the yeast system to improve our understanding of the process. Setoguchi and co-workers reported that integration of tail-anchored proteins is not affected in strains with knocked down TOM components. This method leaves some questions open because one cannot exclude the possibility that minor amounts of the import components, which remained in the depleted strains, were sufficient to support the integration of tail-anchored proteins. In the current study we used mainly knockout strains and thus can exclude this. Interestingly, whereas the membrane insertion of Fis1 was fast and temperature independent in our experimental system, a dependency on temperature and a slower insertion rate were observed by Setoguchi et al. (Setoguchi et al., 2006). We propose that these differences are caused by the variations in the experimental systems used. In our system, the radiolabeled protein is in a diluted solution with direct access to mitochondria, whereas in the earlier study the protein was added to semi-intact cells and had to get access to mitochondria through the crowded cytosol.

Collectively, the specific targeting of TA proteins to mitochondria does not rely on specific interactions with TOM components but rather on other elements. One such element could be the lipid composition of the outer membrane. The ergosterol content in the outer membrane of yeast mitochondria is the lowest among all membranes facing the cytosol. The molar ratio of ergosterol/phospholipid was reported to be 0.01–0.02 in this membrane (Schneider et al., 1999; Zinser et al., 1991). By contrast, this ratio in ER or plasma membranes was reported to be 0.18 and 0.46, respectively (Schneider et al., 1999). Hence, we postulate that the low ergosterol content and the resulting elevated fluidity of the outer membrane help to ensure specific targeting of proteins to the mitochondrial outer membrane. Such a low sterol content can facilitate faster insertion into the membrane of mitochondria and thus can provide mitochondria with a kinetic advantage compared with the other cellular membranes. Substantiating our conclusion is the observation that in our *in vitro* assay, the precursor of yeast Fis1 is inserted at strongly reduced levels to mitochondria isolated from *N. crassa* (C.K., unpublished results). The outer membrane of the *N. crassa* mitochondria contains much higher mol% of ergosterol than the yeast outer membrane (Hallermayer and Neupert, 1974). Interestingly, recent studies reported the localization of a small fraction of Fis1 molecules to peroxisomes (Kobayashi et al.,

2007; Koch et al., 2005). The report that peroxisome membranes also have a low ergosterol content (ergosterol/phospholipid molar ratio of 0.03) (Schneider et al., 1999), adds further support to our proposal that membrane insertion of Fis1 is promoted by a low ergosterol content. Of note, the relatively low cholesterol content of the ER membrane compared with that of other membranes in the secretory pathway was suggested to support the targeting of TA proteins residing in the ER (Brambillasca et al., 2005). However, membrane insertion of mitochondrial TA proteins requires an even lower sterol content. Thus, it seems that a well-defined lipid composition of each subcellular membrane plays a crucial role in keeping the proteomic profile of the organelle membranes. The involvement of lipids such as cardiolipin in stabilization and organization of protein complexes in the inner membrane of mitochondria is well documented (McKenzie et al., 2006; Zhang et al., 2005). However, the current report suggests a new role for lipids in the biogenesis of mitochondria – an involvement in the protein translocation pathway.

How can we explain the *in vivo* targeting of Fis1 to mitochondria? Fis1 was integrated into the membrane of microsomes under cell-free conditions, and without competition from mitochondrial membranes. This observation supports the idea that the ER membrane, if undisturbed by other competing organelles, is capable of inserting tail-anchored proteins that harbor targeting signals for other cellular compartments (Borgese et al., 2007). Thus, as was suggested by Borgese and co-workers, the targeting specificity of TA proteins, which should prevent inappropriate insertion into the wrong organelle, must rely on kinetic factors. These factors that accelerate the irreversible integration into the correct compartment could generate restricted localization by a trapping mechanism. One idea is that the outer membrane of mitochondria contains a yet unidentified protein(s) that enhances the insertion by trapping the Fis1 precursor. Alternatively, the native outer membrane might contain lipid domains or other structural elements that facilitate the insertion of Fis1 and/or stabilize the inserted protein molecules. Such stabilization, which is missing in the lipid vesicles, might not be required for the membrane integration itself but rather can shift the equilibrium between precursor Fis1 to fully inserted Fis1 towards the latter species and thus can result in an enhancement of the overall rate of insertion.

Our proposal does not exclude the potential involvement of cytosolic factors in ensuring the specific delivery of TA proteins to mitochondria. As discussed above, such factors were recently reported to facilitate the integration of some TA proteins into the ER membrane (Abell et al., 2007; Stefanovic and Hegde, 2007). However, factors that mediate the integration of mitochondrial TA proteins remain to be identified. In summary, we propose that Fis1 is inserted into the outer membrane by a novel pathway that does not involve any of the known import components at the outer membrane. A defined lipid composition of the outer membrane contributes to the fidelity of this pathway.

## Materials and Methods

### Yeast strains and growth methods

Standard genetic techniques were used for growth and manipulation of yeast strains. The wild-type strains BY4743, YPH499 and 273-10B were used. Transformation of yeast was carried out according to the lithium-acetate method. Yeast cells were grown under aerobic conditions on YPD [1% (w/v) yeast extract, 2% (w/v) bacto-peptone, 2% glucose] medium. The *tom20* null strain YJTB64 and its corresponding parental strain YJTB4 were utilized [(Lithgow et al., 1994), a kind gift from G. Schatz]. Strains with *tom40* temperature-sensitive alleles were obtained from Dr Kassenbrock (Kassenbrock et al., 1993). The *tom70*, *tom5*, *tom6* and *tom7* deletion strains were obtained from Research Genetics (Huntsville, AL).

## Biochemical procedures

Mitochondria were isolated from yeast cells by differential centrifugation as described (Daum et al., 1982). For isolation of mitochondria from temperature-sensitive mutants and their parental strains, cells were grown at 25°C. Dog pancreas microsomes were purchased from Promega. Radiolabeled precursor proteins were synthesized in rabbit reticulocyte lysate in the presence of [<sup>35</sup>S]methionine (MP Biomedicals) after *in vitro* transcription by SP6 polymerase from pGEM4 vectors (Promega) containing the gene of interest. Blotting to nitrocellulose membranes and immunocytochemistry were carried out according to standard procedures and visualization was by the ECL method (Amersham).

Lipid vesicles were prepared from phospholipids (Avanti Polar Lipids) at a composition similar to that of the mitochondrial outer membrane in yeast (PC, 46%; PE, 35%; PI, 13%; Cardiolipin, 4%; PS, 2%) with or without ergosterol (Fluka). In some cases, 2 mol% Fluorescein-PE (Fluka) was added to the mixture. Lipid films were dried under N<sub>2</sub> atmosphere while cooling. The lipid film was resuspended in a buffer containing 20 mM HEPES, 100 mM NaCl, pH 7.4 and then extruded through a filter with pores with a diameter of 400 nm.

## In vitro protein import and membrane insertion assay

Import experiments were performed in an import buffer containing 250 mM sucrose, 0.25 mg/ml BSA, 80 mM KCl, 5 mM MgCl<sub>2</sub>, 10 mM MOPS-KOH, 2 mM NADH, 2 mM ATP, pH 7.2. Trypsin treatment of mitochondria was performed by adding trypsin (50 µg/ml) for 15 minutes on ice. Trypsin was then inhibited by adding soybean trypsin inhibitor (200 µg/ml) for 5 minutes on ice. To compete mitochondrial import, an excess of precursor protein recombinant pSu9-DHFR (11 µg) was incubated with 50 µg mitochondria for 5 minutes at 25°C before the import reaction. Mitochondria isolated from strains containing a temperature-sensitive variant of Tom40 or from the corresponding parental strain were incubated at 37°C for 15 minutes before the import reaction.

Labeling with IASD was performed according to a published procedure with some modification (Kim et al., 2004). Radiolabeled proteins in 5 µl reticulocyte lysate were incubated for 30 minutes at 25°C or for various time points at 0°C with either isolated mitochondria or lipid vesicles. Mitochondria or vesicles were re-isolated by centrifugation (10 minutes, 13,200 g, 2°C for mitochondria or 60 minutes, 186,000 g, 2°C for vesicles). The mitochondria and the vesicles were resuspended in labeling buffer (import buffer supplemented with 50 mM Tris-HCl pH 8.0, 4 M urea, 1 mM DTT) and IASD (10 mM) was added for further 20 minutes at 25°C. The labeling reaction was stopped with the addition of 200 mM DTT. The mitochondria and the lipid vesicles were diluted with 600 µl import buffer containing 120 mM KCl, reisolated as above and resuspended in sample buffer. Carbonate extraction in combination with gradient centrifugation was performed as described (Rapaport et al., 2001).

## Recombinant DNA techniques

For construction of Fis1 variants, the *FIS1* open reading frame was amplified by PCR from genomic yeast DNA. The resulting product was digested with *EcoRI* and *HindIII* and cloned into the *EcoRI/HindIII* sites of pGEM4 vector (Habib et al., 2003). The GFP-Fis1(TM) construct was obtained by first inserting (using a Stratagene site-directed mutagenesis kit) a *SacI* site between the transmembrane and the cytosolic domains of Fis1. Then the DNA fragment coding for the cytosolic domain of Fis1 was cut out and a PCR-amplified GFP coding fragment was inserted in this site. The Fis1-TMC variant was constructed by site-directed mutagenesis using a site-directed mutagenesis kit (Stratagene). For expression in yeast cells, the Fis1 variants were introduced into the multicopy yeast plasmid, pYX132.

## Fluorescence microscopy

Microscopy images were acquired with an Axioskop20 fluorescence microscope equipped with an Axiocam MRm camera using the 43 Cy3 filter set and the AxioVision software (Zeiss). To stain mitochondria, cells were transformed with a yeast expression vector encoding mitochondrially targeted DsRed.

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