

**Bacterial tail anchors can target to the mitochondrial outer membrane and to
peroxisomes**

by

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This thesis is dedicated to peace

ABSTRACT

Mitochondria are double membrane bound organelles, surrounded by inner and outer membranes. Mitochondria are unique, having their own genetic material and a complete translation system. Yet, most of the mitochondrial proteins are produced in the cytosol, as a consequence of the evolutionary origin of mitochondria. Accordingly, an α -proteobacterial endosymbiont within the eukaryotic cell was transformed into the mitochondrion and reduced its genomic size by transferring most of its genes to the host nucleus. Translocation of such nuclear encoded proteins to the mitochondria requires special translocases found both at the inner and the outer membrane. As the conserved mitochondrial translocase of the outer membrane (TOM) complex seems to require pre-existing TOM complexes for membrane insertion, evolution of the initial translocon for re-targeting those proteins back to mitochondria is still a matter of debate. In order to find the possible bacterial origins of mitochondrial translocation machinery, based on a previous survey in *Escherichia coli* proteome, we studied self-inserting, tail-anchored bacterial polypeptides that might have formed the initial gate allowing proteins to access mitochondria from the cytosol. Indeed, predicted tail anchor (TA) sequences of bacterial DUF883 family proteins, including ElaB and YqjD TAs, were targeted and inserted at the mitochondrial outer membrane within the budding yeast *Saccharomyces cerevisiae*. In addition, we also found that the YgiM TA localized to yeast peroxisomes. Our results support the view that eukaryotic cells are able to process membrane targeting signals of bacterial proteins acquired via lateral gene transfer, enabling us to suggest a scenario in which mitochondrial protein translocation was initially allowed by tail-anchored proteins.

ÖZET

Mitokondriler iç ve dış membranlar tarafından çevrili, çift membranlı organelldir. Mitokondriler, kendi genetik materyali ve translasyon sistemi olan, benzersiz organelldir. Buna rağmen, mitokondrinin evrimsel sürecinin bir sonucu olarak, mitokondri proteinlerinin çoğu sitoplazmada üretilir. Buna göre, ökaryotik hücrenin içinde bulunan endosimbiyont bir alfa-proteobakteri, mitokondriye dönüşmüş ve genlerinin büyük bir kısmını içinde bulunduğu hücrenin çekirdeğine aktararak genomik boyutunu azaltmıştır. Bunun gibi hücre çekirdeğinde sentezlenen mitokondri proteinleri mitokondriye ulaşmak için iç ve dış membranda bulunan translokazlara ihtiyaç duyarlar. Mitokondri dış membranında bulunan translokaz (TOM) kompleksinin membrana yerleşmesi için hali hazırda yerleşmiş TOM komplekslerine ihtiyaç olduğundan, bu proteinleri tekrar mitokondriye iletecek translokazın nasıl evrimleştiği hala tartışılmaktadır. Mitokondri translokasyon sisteminin muhtemel bakteriyel kökenlerini bulmak adına, daha önceden *Escherichia coli* proteomunda yapılmış bir incelemeden yola çıkarak, ilk translokonu oluşturup proteinlerin sitoplazmadan mitokondriye erişimini sağlamış olabilecek, kendi kendine membrana yerleşebilen, tail-anchored (TA) bakteriyel polipeptitlerle çalıştık. Gerçekten de, DUF883 ailesine ait ElaB ve YqjD proteinlerinin tahmin edilen TA sekansları *Saccharomyces cerevisiae* tomurcuklanan maya türünün mitokondri dış membranına yerleşip entegre oldular. Buna ek olarak bakteriyel YgiM proteinin TA sekansının da maya peroksizomlarına yerleştiğini gözlemledik. Bu sonuçlar ökaryotik hücrelerin yatay gen transferiyle edindikleri bakteriyel proteinlere ait membran entegrasyon sinyallerini işleyebildiklerini kanıtlayıp, mitokondri protein translokasyonunun ilk etapta TA proteinler tarafından sağlandığı senaryosunu önermemizi sağlamaktadır.

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NOMENCLATURE

AAA	ATPases associated with diverse cellular activities
AβPP	Amyloid β precursor protein
b5	Cytochrome b5
COX	Cytochrome oxidase
EGT	Endosymbiotic gene transfer
ER	Endoplasmic reticulum
ERMES	ER-mitochondria encounter site
GET	Guided entry of TA proteins
HGT	Horizontal gene transfer
HEK	Human embryonic kidney
MOM	Mitochondrial outer membrane
mtDNA	Mitochondrial DNA
OMb	Outer mitochondrial membrane cytochrome b5
PEX	Peroxin
PMP	Peroxisomal membrane protein
PTS	Peroxisomal targeting sequence
rRNA	Ribosomal RNA
SAM	Sorting and assembly machinery
SEC	Secretory
sfGFP	Superfolder GFP
SNARE	Soluble NSF (N-Ethylmaleimide-Sensitive Factor) attachment protein receptor
SND	SRP-independent targeting
SRP	Signal recognition particle
TA	Tail anchor

TIM	Translocase of the inner membrane
TMD	Transmembrane domain
TOM	Translocase of the outer membrane
TRC	Transmembrane recognition complex
tRNA	Transfer RNA
WT	Wild-type



Chapter 1

INTRODUCTION

Mitochondrial matrix is surrounded by inner and outer membranes. The inner membrane forms tubular invaginations called cristae and contains enzyme complexes that function in oxidative phosphorylation ¹. The outer membrane is porous, allowing the passage of ions and small, uncharged molecules through porins. On the other hand, the inner membrane stands as a diffusion barrier against ions and other molecules, and this ion selectivity generates an electrochemical membrane potential. Larger proteins are guided through special translocases found both in the inner and the outer membrane and are imported into the mitochondria ². Even though mitochondria have their own genome and a complete translation system, most of the mitochondrial proteins are produced in the cytosol and pass through these translocases ³. According to an evolutionary point of view, an α -proteobacterial endosymbiont within the eukaryotic cell was transformed into the mitochondrion and decreased its genomic size by transferring most of its genes to the host nucleus ^{4,5}, a process called endosymbiotic gene transfer (EGT). While some of these proteins acquired new functions in other subcellular locations, a minor portion of these proteins were still functional in mitochondria ⁵. In addition to such proteins, proteins acquired through horizontal gene transfer (HGT) by the host nucleus from other organisms contributed to the mitochondrial proteome. Also, already existing host proteins acquired new functions in mitochondrial biogenesis ⁶. All such proteins were needed to re-target to the proto-mitochondria ^{5,7,8}. Thus, evolution of a translocon for targeting those proteins to mitochondria was needed. As the assembly of the conserved mitochondrial translocase of the outer membrane (TOM) complex requires pre-existing TOM complexes ^{9,10}, the question

of how the initial translocon has evolved remains unanswered. While their ancient origins are still to be explored, most of the components of fungal mitochondrial import machineries have already been discovered. Thus, studies of the mitochondrial protein import in eukaryotic organisms that are higher than fungus is important. Such studies especially in mammalian systems, are essential for revealing import components that are previously unidentified ¹¹.

Aiming to find the possible bacterial origins of mitochondrial translocation machinery, we took advantage of self-inserting, tail-anchored (TA) bacterial polypeptides that might potentially have initiated the formation of the first gate for protein access into the mitochondria from the cytosol, a necessary step for the evolution of modern mitochondria, based on a previous survey in *Escherichia coli* proteome. Predicted TA sequences of DUF883 family proteins ElaB and YqjD were strongly localized to and inserted at the mitochondrial outer membrane (MOM) within the budding yeast *Saccharomyces cerevisiae*. Moreover, the YgiM TA localized to yeast peroxisomes, an organelle exclusively responsible for metabolism of fatty acids in yeast and contributing the mitochondrial fatty acid degradation in human cells. Our results support the notion that eukaryotic cells are able to process membrane targeting signals of bacterial proteins obtained by lateral gene transfer. This allows us to suggest a scenario for the evolution of mitochondrial protein import in which mitochondrial protein translocation was initially driven by tail-anchored proteins. Furthermore, the mCherry fluorescent protein fused to YgiM TA showed strong localization to peroxisomal membranes in different mutant strains with less artifact compared to other markers tested. Accordingly, we propose that YgiM TA can be used as a functional peroxisomal and a pre-peroxisomal marker. As the protein domain itself has no intrinsic function in the organelle, it interferes less with the peroxisomal biogenesis compared to other peroxisomal targeting peptides. Thus, YgiM TA can serve as a functional tool to study the peroxisomal trafficking and protein import in different *S. cerevisiae* mutants. Additionally, we suggest a genetic selection method for mammalian cells that are unable to operate mitochondrial protein import, a system that could serve great value for finding novel mitochondrial import components in mammalian systems, to have a better understanding of mitochondrial biogenesis in higher eukaryotes. Once we establish a functional selection method, we will generate a mammalian cell population with possible mitochondrial import defects using genome-wide knockout or knockdown strategies and perform genetic selection

to search for novel mitochondrial import components. Lastly, we generated mammalian cells expressing a commercially available fluorescent biosensor that is used for following intracellular energy status depending on cellular ATP:ADP ratio. Once we confirm the functionality of this cell line for detecting changes in cellular ATP and ADP levels, we may use it to examine the metabolism of mammalian mitochondria.

Chapter 2 provides a literature review about eukaryogenesis and the origins of mitochondria. It briefly explains the endosymbiotic theory, mitochondrial protein import machineries, and discusses the potential role of TA proteins in the evolution of protein translocons. Moreover, it reexamines the protein trafficking in peroxisomes and discusses the pathways that facilitate the insertion of peroxisomal membrane proteins with a special emphasis on TA proteins.

In Chapter 3, we demonstrate that bacterial TAs ElaB and YqjD can insert and function at the MOM when the TA of a MOM protein is replaced with either of them. A likely scenario suggested for the evolution of the initial mitochondrial translocon is explained based on these findings.

In Chapter 4, we demonstrate that the TA of another bacterial protein YgiM, can also insert at peroxisomal membranes and show functionality when it replaces the TA of a peroxisomal membrane protein, Pex15p in *S. cerevisiae* cells. We show that YgiM TA can localize to pre-peroxisomal and mature peroxisomal membranes in different mutant strains and can be used to track peroxisomal trafficking in yeast cells.

Chapter 5 presents our progress for establishing a selection system to isolate mammalian cells with mitochondrial import defects.

Chapter 6 reports our very preliminary data for generating a mammalian cell line to follow energy status of cells by using a commercially available fluorescent biosensor.

Chapter 2

LITERATURE REVIEW

2.1 Overview of eukaryogenesis and origins of mitochondria

The theory of the origin of eukaryotic cells suggests that two of the eukaryotic organelles, the mitochondria and the plastids of the photosynthetic organisms were once free-living organisms of α -proteobacterial ancestry¹². The early anaerobic atmosphere of the world gave rise to the evolution of anaerobic bacteria and later to photosynthetic organisms such as photosynthetic bacteria. Eventually, formation of the aerobic atmosphere allowed development of an aerobic metabolism in prokaryotes¹³. According to one of the recent models¹⁴, an epibiotic α -proteobacterium, the proto-mitochondrion, was initially residing on the surface of an archaeal host. Due to an increasing mutualistic association between the two organisms, the host formed membrane protrusions and bleb enlargements so that the organisms could interact through a larger area. These structures eventually formed the outer nuclear membrane, plasma membrane, cytoplasm, and the ER whereas the α -proteobacterium was permanently incorporated into the host and finally refashioned into the eukaryotic organelle, mitochondria¹⁴. The endosymbiont is predicted to be an aerobe¹⁵ or a facultative anaerobe that could perform fermentation in the absence of oxygen or respire under aerobic conditions¹⁶. Slightly later, after a similar symbiotic relation between a photosynthetic bacteria and an early protozoa, the photosynthetic endosymbiont was transformed into photosynthetic plastids to form the eukaryotic algae¹⁷. However, the focus of this dissertation will be the evolution of mitochondrion and its protein targeting system.

2.1.1 Origins of mitochondrial genome

The initial evidence underlying the theory of mitochondrial evolution was the discovery of a separate genetic material within this organelle. In 1950s, studies made with yeast metabolism that focus on respiratory enzymes and the subcellular units from which they are synthesized implicated that mitochondria might have its own DNA ^{18,19}. Consistently, “mitochondrial fibers” that could be digested by deoxyribonuclease were found in 1963 ²⁰. These observations were also confirmed by an independent study in which DNA was observed in purified yeast mitochondria as a separate genetic material within this organelle ²¹. Finally, it was confirmed that the mitochondria have their own translation system allowing protein synthesis inside the mitochondria distinct from the cytosolic protein translation machinery ²². Developing sequencing and DNA cloning technologies allowed researchers to confirm bacterial origins of the mitochondrial genetic material through detailed comparison between mitochondrial DNA (mtDNA) and bacterial genome ²³. After this discovery, it was possible to consider the bacterial origin to which mitochondria is most closely related.

The function of mtDNA was revealed after its complete sequencing in mammalian species. Accordingly, mtDNA that is around 16-kb was reported to encode subunits of electron transport chain, ATP synthase, the transfer RNA (tRNA), and ribosomal RNA (rRNA) components of the mitochondrial protein synthesis system ²⁴. Similar to the nuclear genome, the genomic size of mtDNA as well as the physical, organizational features, and modes of gene expression remarkably varies within the eukaryotic domain ²⁵. More interestingly, mitochondrial genome in some of the non-animal species reduced dramatically in size, and many of the protein or tRNA genes encoded in the animal mtDNA were lost. The mtDNA of *Plasmodium falciparum*, the smallest known mtDNA with a size of around 6 kb, encodes only three protein and lacks any of the tRNA genes ²⁶. On the other hand, in some of the non-animal eukaryotes, mtDNA seems to have a larger proteome, containing additional respiratory or ribosomal proteins ⁶. Notably, mtDNA size of land plants has considerably increased, reaching a size of 11,000 kb that is even larger than a bacterial genome ²⁷. It is obvious that mtDNA has encountered significant re-arrangements decreasing or increasing its genomic size and does not resemble any of the current bacterial genome regarding its genetic organization or protein expression ⁶.

2.1.2 Origins of mitochondrial proteome

Mitochondrial proteome is derived from a variety of different sources including the proteome of the host, the endosymbiont, or other external organisms ²⁸. Thus, origins of mitochondrial proteome varying from a low of three proteins in *P. falciparum* parasite, to a high of 66 in *Andalucia godoyi*, a species of jakobids, illuminate how an aerobic bacterial endosymbiont might have transformed into the modern mitochondrion ⁴. Although mitochondria have their own genome and a complete transcription-translation machinery, the vast majority of the mitochondrial proteins are encoded by the nucleus ²⁹. Not only the mtDNA encoded mitochondrial proteins, but also nucleus encoded proteins that are imported to mitochondria are considerably variable between different species. The estimated number of mitochondrial proteins is several hundred in *P. falciparum* while this value exceeds 3000 in vertebrates ³⁰. The diversity of mitochondrial proteins involves addition of novel proteins through horizontal gene transfer (HGT) or new functions acquired by the already existing host proteins ^{6,28}. Alternatively, some of the nuclear encoded mitochondrial proteins are derived from the endosymbiont through gene transfer to host nucleus, a process called endosymbiotic gene transfer (EGT). Also, some of the endosymbiotic proteins were lost due to the substitution of a functional endosymbiotic gene by a nuclear homolog or loss of functionality of a gene product during the course of evolution ³¹. Most of the protein products of the genes that are transferred to the host nucleus acquired new functions in other subcellular fractions and only a minor portion was re-targeted back to the organelle ⁵. In addition to these α -proteobacteria-derived proteins, products of the genes acquired through HGT and proteins evolved *de novo* within the eukaryotic cell needed to target the mitochondria ^{5,7,8}. This requires their recognition in the cytosol, translocation through the mitochondrial membranes, and assembly inside the mitochondria, a series of conserved events that take place in modern eukaryotes ³². However, such a protein-targeting machinery does not seem to have counterparts in bacteria, suggesting that they have formed *de novo* ³³. Furthermore, the main translocation complex of the current mitochondria requires pre-existing components for its insertion, making it challenging to estimate the evolution of the initial translocon ⁹.

2.2 Protein import machineries in modern mitochondria

Protein precursors encoded by the nuclear genome are imported to the mitochondria depending on the signal sequences called presequences. These presequences are generally found at the N-terminus of the precursor protein³⁴, although mitochondrial targeting depending on a targeting domains found at the internal³⁵ or C-terminal region of the protein is also possible³⁶. Most of the mitochondrial proteins are initially recognized by the MOM receptors Tom20 and Tom70. Upon recognition, proteins are translocated through a common gate called the translocase of the outer membrane (TOM) complex. Once the proteins are translocated across this outer membrane translocon, they are recruited to other mitochondrial import machineries, depending on their final destination. If the protein is an intermembrane space resident protein, it is recruited to the mitochondrial intermembrane space import and assembly (MIA) machinery that drives protein import into the intermembrane space. On the other hand, integral proteins of the outer membrane, such as β -barrel proteins, are guided by the sorting and assembly machinery (SAM) complex to be inserted to the outer membrane while inner membrane integration of mitochondrial metabolite carrier proteins is driven by the membrane potential through the translocase of the inner membrane 22 (TIM22) complex. Finally, presequence containing proteins are recruited directly to the translocase of the inner membrane 23 (TIM23) complex upon their exit from TOM. After their passage to TIM23 complex, they are either laterally sorted to the inner mitochondrial membrane, again depending on the membrane potential, or in imported into the matrix space through the presequence translocase-associated import motor (PAM), a process that is driven by ATP. When translocation to the matrix space is completed, the protein presequence is cleaved by the mitochondrial processing peptidase (MPP)³⁷.

TOM complex, the common translocon of the mitochondrial outer membrane (MOM), is very much conserved among varied species. Although TOM complex is the main gate allowing protein import into the mitochondria, the insertion of the core components of this translocon takes place through the TOM complex itself⁹. Tom40, the most abundant element of the TOM complex³⁸, is the pore forming subunit, constituting the hydrophilic channel that allows import of the preproteins³⁹. Interestingly, the core component Tom40 is inserted and assembled to the MOM in an N-terminus dependent manner through pre-existing TOM complexes⁹. Thus, one

could imagine that for the formation of such a protein importing machinery in the course of mitochondrial evolution, the first translocator may have been a self-inserting protein ⁴⁰.

2.2.1 Theories for the insertion of an early translocon

Similar to the β -barrel TOM complex of the MOM ⁹, the α -helical protein channels found on bacterial inner and plasma membranes such as YidC/Oxa/Alb3 family, or the SecYEG/Sec61, protein families that mediate protein translocation ⁴¹⁻⁴³, require already existing translocon channels for their insertion into lipid bilayers. As these channels are α -helical proteins themselves, it is an evolutionary problem to explain the insertion of such translocons without an already existing translocon to assist their insertion into the lipid bilayer ⁴⁰. Although it is not an α -helical protein, insertion of the initial translocon that would later evolve to the current TOM complex at the MOM resembles a similar evolutionary problem. How did the first translocon insert at early mitochondrial membranes?

There is no evidence for spontaneous insertion of any of the known translocons. YidC is a bacterial inner membrane translocon that assists the insertion of other integral membrane proteins as an independent membrane protein insertase, as well as in cooperation with SecYEG complex that constitutes the major route of membrane insertion on bacterial membranes. YidC, the homolog of mitochondrial inner membrane translocating protein Oxa1p, inserts at bacterial inner membrane through pre-existing YidC along with the components of the SEC pathway, SecA and SecY ^{42,44}. Eukaryotic translocons also insert into lipid bilayers through other translocons. The eukaryotic homolog of YidC, Oxa1p requires pre-integrated Oxa1p ⁴³ or TIM22 requires pre-integrated TIM22 ⁴⁵ for their insertion at the mitochondrial inner membrane. All of these proteins are found on these membranes because they are inherited to the progeny by the division of pre-existing organelles or through membranes of the mother cell ⁴⁰. Thus, it is likely that progenitors of the integral membrane proteins should have inserted at the lipid bilayer through a different mechanism ⁴⁰.

One possible mechanism for the unassisted insertion of membrane proteins could be through a membrane fusion process. For instance, the archaeal proton pump bacteriorhodopsin can be integrated into unilamellar dimyristoyl phosphatidylcholine vesicles in vitro after its initial incorporation into archaeal membrane fragments ⁴⁶. However, this process requires an earlier

integration of the protein into smaller membranes. Proteins with single transmembrane helices can also be inserted at lipid bilayers in the absence of an earlier translocon, with the help of a chaperone that would correlate with the hydrophobicity of the membrane passing domain of the protein. Accordingly, TA proteins can be inserted to the ER membrane by Hsp40/Hsc70 chaperone activity in an ATP dependent manner ⁴⁷. Alternatively, protein insertion at cellular membranes might even be independent of the any known intracellular factors. TA proteins ^{48,49} or peptides produced by organisms as part of a defense mechanism ^{50–52} can spontaneously insert at lipid membranes. Such self-inserting proteins might have functioned as a chaperone to initiate the integration of the early translocon into the lipid or few of these proteins might have cooperatively formed a channel inside the lipid bilayer that would mediate lateral insertion of the early translocon ⁴⁰.

2.2.2 Self-inserting antimicrobial peptides

If all known translocons require already integrated translocons to insert at the membranes that they are destined for, the first protein that eventually evolved into the conserved protein gate of the MOM might have inserted spontaneously at the early mitochondrial membrane. Although none of the modern translocons are able to spontaneously insert into lipid bilayers because of the hydrophobic nature of the protein, there are other peptides that favor spontaneous insertion at plasma membrane as well as at intracellular membranes of the eukaryotic cell ⁴⁰.

There are antibiotic peptides of fungal or eukaryotic origin that can facilitate spontaneous insertion on membranes. Alamethicin is an antibiotic peptide produced by the fungus *Trichoderma viride* and can spontaneously insert into lipid bilayers at high concentrations that allow enough membrane deformation energy ⁵⁰. There are such peptides produced by higher eukaryotes as well, including melittin, the bee venom that can self-insert into a lipid bilayer, depending on the lipid to peptide ratio and other environmental conditions ⁵¹. Also, a group of antibiotic peptides, the magainin family, that are found in the frog skin are known to act on membranes through electrostatic interactions ⁵² and initiate pore formation ⁵³. However, it is unlikely that one of these peptides could serve as the origins of the early translocator as most of these antibiotic peptides are exclusive to the eukaryotic lineage ⁴⁰. There are similar peptides of prokaryotic origin including certain bacteriocins. Curvacin A, a member of the Class IIa Bacteriocin can also insert to the hydrophobic core of the membrane depending on the glycine

residues found on the C-terminal helix⁵⁴. Similarly, colicin, a type of bacteriocin produced by some of the *E. coli* strains has pore-forming capacity. After the toxin is released to the environment, it forms an ion-conducting channel at the inner membrane of the target bacteria upon rearrangements of the α -helices found at the C-terminal globular water-soluble domain⁵⁵.

These antimicrobial peptides seem to be suitable candidates for being the descendents of a progenitor MOM translocon, with their spontaneous insertion mechanisms and pore forming capabilities. Alternatively, a different group of proteins, certain members of which can readily insert at the MOM without the assistance of any known mechanisms could have played a key role for the evolution of a translocator at the MOM throughout the eukaryogenesis.

2.3 Tail-anchored (TA) proteins

Tail-anchored proteins are a group of proteins containing a transmembrane domain near the C-terminus. They are integrated into the lipid bilayer via this single hydrophobic patch of amino acids at the C-terminus, and the N-terminus is oriented on the cytosolic face of the membrane⁵⁶. Because of their natural membrane topology that locates the N-terminus on the cytosolic face, their co-translational insertion on the endomembrane system through signal recognition particle (SRP) dependent Sec61 translocon is not possible⁵⁷. Thus, C-terminal TA proteins follow different insertion mechanisms on their way to secretory system or autonomous organelles.

2.3.1 Mechanisms for TA protein targeting and insertion

The insertion mechanism of a TA protein is dependent on its final destination. If a TA protein is destined for the secretory pathway, it is first targeted to ER and then directed to its ultimate destination via vesicular transport. These proteins such as giantin an integral component of the Golgi membrane, could be destined for the Golgi apparatus⁵⁸ or for plasma membrane like the yeast SNARE-type protein Sso2p⁵⁹. Although peroxisomes multiply by growth and division⁶⁰, they are not autonomous⁶¹. There is evidence for the tail-anchored peroxisomal membrane protein Pex15p to be inserted at peroxisomal membrane following an ER to peroxisome route⁶². Because of the membrane orientation of the TA proteins, placing the N-terminus at the

cytosolic face, TA proteins are suggested to follow post-translational pathways to be inserted at their target membrane ⁶³.

Sec61 is the main translocon found at the ER membrane and is associated with both co-translational SRP (signal recognition particle)-dependent import ⁵⁷ and posttranslational import that depends on cytosolic heat-shock protein chaperones and Sec63 complex to recognize the signal sequence ⁶⁴. Thus, possible role of Sec61 in TA insertion at ER is well studied. Experiments made with mammalian ⁶⁵ and fungal ⁶⁶ systems indicated that TA proteins can insert into Sec61-depleted microsomes in an ATP dependent manner, suggesting at least one other alternative machinery is involved in this process. On the other hand, crosslinking studies later represented a weak association between the ER SNARE protein synaptobrevin and components of SEC machinery ⁶⁷, referring to partial involvement of the Sec61 translocon through the SRP independent post-translational pathway. A recent, study suggests that this association might occur with the help of an alternative pathway called SRP-independent targeting (SND) pathway. Involvement of three new proteins named Snd1p, Snd2p, and Snd3p, in protein targeting at the ER membrane has been suggested ⁶⁸.

The existence of an alternative pathway that inserts TA proteins at the ER membrane became apparent after the discovery of the cytosolic chaperone that was named transmembrane recognition complex subunit of 40 kDa (TRC40) in mammals ⁶⁹. In parallel, through an epistatic miniarray profile in yeast, existence of three uncharacterized genes that showed strong genetic interactions was revealed. Initially predicted to function in Golgi-ER trafficking, these three proteins were referred as GET complex ⁷⁰. Later the function of this GET complex in insertion of TA proteins into the ER membrane was characterized, and the meaning of GET was changed to “Guided Entry of Tail-anchored proteins”. Accordingly, Get3p was discovered to function in recognition of TA proteins in the cytosol, homologous to mammalian TRC40, and recruit the cargo protein to Get1p/Get2p receptor on the ER membrane to facilitate its membrane insertion. Recognition by Get3p acts as a decisive step for the protein targeting to the ER, and its deletion mislocalizes the ER destined TA proteins to mitochondria, while absence of Get1/Get2 receptor results in sequestration of these TA proteins into cytosolic aggregates upon interaction with the Get3p ⁷¹. In this context, GET pathway is represented to be specific for secretory TA proteins and do not interact with MOM TA proteins ^{71,72}. SND pathway has been reported to compensate for the loss of Get3p as well suggesting that it constitutes an alternative also for GET pathway

⁶⁸. The role of SND pathway in ER protein targeting in collaboration with both SEC and GET pathways is depicted in Figure 2.1.

There are integral ER TA proteins suspected to be independent of any known ER membrane components for their membrane insertion. One such protein is ER targeted cytochrome b5 (b5) that can insert both at ER membranes and mitochondrial derived microsomes *in vitro* ⁷³, and this process was reported to be independent of any membrane proteins, but rather affected by the cholesterol levels of the membrane ⁷⁴. Later, the possible assistance of any cytosolic chaperones or other soluble proteins in b5 insertion was excluded, with *in vitro* experiments using b5 constructs translated in cell-free systems or bacteria. In fact, cytosolic proteins that inhibit b5 insertion into liposomes were identified ⁷⁵. Thus, insertion of b5 at the ER membranes seems to be unassisted by any membrane or cytosolic proteins.

Although it has been hypothesized that an ER to mitochondria protein transfer might be taking place through ER-mitochondria encounter site called ERMES ⁷⁶, there had been no evidence suggesting that TA proteins destined for mitochondria follow the secretory pathway on their way to their final location. However, a recent study suggests that the ER membranes contain tail-anchored mitochondrial DRP1 receptors FIS1 and MFF that recruit DRP1 to the ER membrane in human cells ⁷⁷. Moreover, DRP1 was also shown to be recruited to mitochondria from the ER and mediate mitochondrial division, suggesting that there could be an ER to mitochondria translocation mechanism for tail-anchored proteins. As these proteins also localize to peroxisomes, their possible recruitment also to the peroxisomal membrane remains to be investigated.

Bcl-2 protein family contains many TA proteins that antagonistically act on intrinsic apoptosis pathway ⁷⁸. One such example is Bax, the pro-apoptotic Bcl-2 family protein. Similar to antimicrobial peptides, Bax permeabilizes the MOM, oligomerizes to initiate pore formation, and commits cell death ⁷⁹. Another member of the Bcl-2 protein family Bak was reported to insert at the MOM independent of the TOM complex. When the core component of the TOM complex, Tom40, was depleted in mammalian cells, insertion of fluorescently tagged Bak protein at the MOM was not affected, while the import of matrix-destined preproteins or MOM-destined β -barrel proteins was heavily compromised. Interestingly, MOM insertion of the full-length Bak but not the GFP fused to the MOM-targeting signal of Bak was affected by the knockdown of VDAC2, the binding partner of Bak, suggesting that the MOM-targeting signal of Bak is masked within the full-length Bak, and VDAC2 functions to unmask the targeting

signal⁸⁰. Finally, targeting of anti-apoptotic Bcl-XL protein to the MOM is heavily dependent on the C-terminal transmembrane domain⁸¹, and the insertion at the MOM occurs via folding and unfolding cycles that continues throughout the course of insertion⁸².

Another MOM TA protein apart from the Bcl-2 family is Fis1p, the conserved protein that takes role in mitochondrial and peroxisomal fission events⁸³. Fis1p is one of the best studied TA proteins, regarding its insertion mechanism, and yet, none of the known mitochondrial insertion pathways seems to take role in its MOM integration⁴⁸.

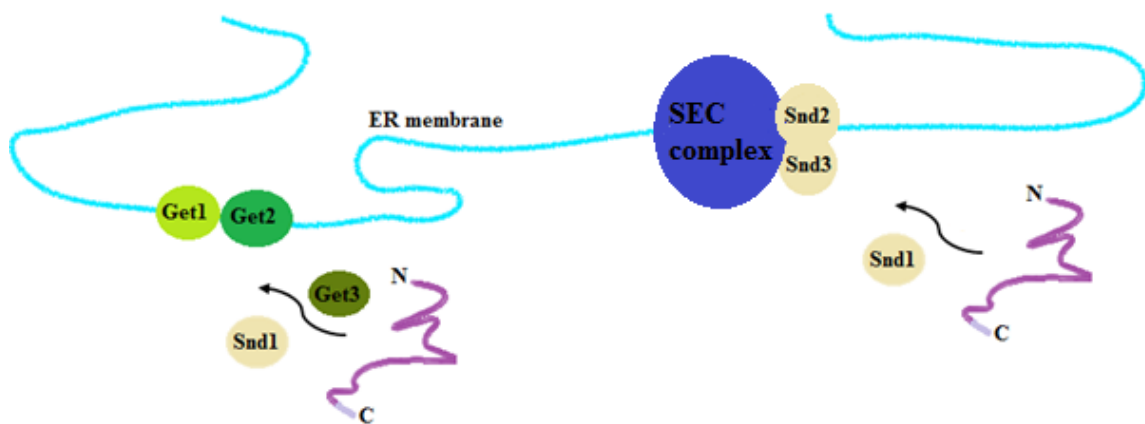


Figure 2.1 SND pathway works in collaboration with both SEC and GET pathways for TA protein targeting at the ER membrane. Snd1p recruits TA proteins to the Get1/Get2 complex found on the ER membrane. Alternatively, Snd1 recruits TA proteins to SEC/Snd2/Snd2 complex also at the ER membrane. Either of these pathways functions in targeting TA proteins to the ER.

2.3.2 Transmembrane domain as the determinant of membrane insertion

TA proteins can be destined for different intracellular locations including ER, Golgi apparatus⁵⁸, peroxisomes⁸⁴, plasma membrane⁵⁹, or mitochondria⁸⁵. As mentioned earlier, most of these destinations, except for mitochondria, are part of the endomembrane system and TA proteins first insert at ER membrane on their way to these different compartments while autonomous organelles such as mitochondria, also plastids in plants, import TA proteins directly from the cytosol. On the other hand, import of peroxisomal TA proteins seems to differ

between fungal and mammalian systems. While tail-anchored PMPs in yeast can be targeted to peroxisomes directly from the cytosol or through ER via GET pathway ⁷¹, TA protein targeting to mammalian peroxisomes seem to depend on direct pathway from the cytosol, upon protein interaction with PEX19 ⁸⁶. Thus, the final location of a cytosolic TA protein is primarily dependent on its discrimination between these different membranes ⁵⁶.

The main determinant whether a TA protein will insert at the ER or the mitochondrial membrane is generally its transmembrane domain (TMD) and the amino acids at the flanking region after the TMD on the C-terminal ⁵⁶. While plant cytochrome b5 dual localizes at mitochondrial and ER membranes, the C-terminus of cytochrome b5 provide organelle specificity in mammalian cells ⁸⁷. Thus, MOM protein cytochrome b5 (OMb) and its isoform ER-localized cytochrome b5 (b5) are of great value to study the features that discriminate between the two membranes. Mutation experiments made with these two proteins revealed that deleting approximately 10 amino acids from the C-terminus of OMb mislocalizes it to the cytosol, while replacing the corresponding region of ER destined b5 with the 10 amino acids residues of OMb localizes b5 to mitochondria in mammalian cells. Mutating the positively charged amino acids to a neutral one at C-terminus of OMb also shifts mitochondrial distribution of OMb mainly to ER ⁸⁸. Similarly, a splice-isoform of vesicle-associated membrane protein-1 (VAMP-1A), named VAMP-1B has a hydrophobic TMD shortened by four amino acids at the C-terminus, and this region is immediately followed by three charged amino acids that are absent from VAMP-1A. These additional features of the isoform VAMP-1B allows protein to target mitochondria while VAMP-1A is localized to plasma membrane or intracellular vesicles ⁸⁹. Thus, a relatively shorter hydrophobic region and presence of positively charged amino acids at the flanking C-terminus are generally the main determinants that target a TA protein to the MOM.

As mentioned earlier, while most TA proteins targeting the ER require energy and assistance of other proteins for membrane insertion, b5 seems to insert at the ER membrane without the assistance of any known cellular factors. Thus, the unique features that allow unassisted integration of b5 at biological membranes is an area of interest. With the help of chimeric protein constructions, it was revealed that hydrophobic TMD of the protein is responsible for this unique insertion. Replacing the TMD of synaptobrevin 2 (Syb 2), a well-studied ER TA protein known to require assistance for insertion ⁶⁵, with b5 TMD, converted Syb 2 to a protein capable of unassisted insertion while b5 was converted to be an assistance-dependent protein

when its TMD was replaced with Syb 2 TMD. Moreover, increasing the hydrophobicity of b5 TMD inhibited the unassisted insertion of the protein, while decreasing that of Syb 2 allowed its unassisted insertion ⁴⁹.

Unlike MOM proteins, length and hydrophobicity of the TMD or charge of the amino acids at the flanking region do not affect the initial acceptability of the protein at the ER membrane ⁶³. In fact, artificial TMDs as short as 12 amino acids with overall hydrophobicity can target TA proteins to microsomes *in vitro* with no sequence specificity at this region ⁹⁰. However, the length of the TMD can affect the final destination of the protein within the secretory pathway. When ER-localized *Saccharomyces cerevisiae* protein Ubc6p is expressed in mammalian cells, it also localizes to the ER, and lengthening the hydrophobic segment re-locates the protein to Golgi complex while further increasing the length of this region locates the protein to plasma membrane ⁹¹.

The targeting information of TA proteins also seems to be conserved within different species. For instance, the glycine residues found on the TMD of yeast MOM protein Fis1p, along with the positive charges at the C-terminus are conserved between different fungal species, plants, and human ⁹². Furthermore, although yeast Pex15p does not compensate the loss of its mammalian homologue PEX26 when expressed in mammalian cells, it still localizes to mammalian peroxisomes ⁹³. In the same study, mammalian PEX26 localizes at yeast peroxisomes although it does not functionally replace Pex15p.

A recent study revealed the unique characteristics of Fis1p TMD that play a role in its mitochondrial targeting in *S. cerevisiae* cells. In this work, the capability of the TMD for accepting different amino acids without compromising the MOM targeting of the protein was examined with deep mutational scanning. According to this screen, mutations that convert neutral amino acids to positively charged lysine or arginine were more acceptable at the TMD than expected ⁹⁴. Presence of positively charged amino acids at the very carboxyl terminus of mitochondrial TA proteins is known to be required for efficient MOM insertion by ensuring organelle specificity and preventing ER localization ^{36,88,89,95}. However, it is thought to be energetically unfavorable for the TMD that is embedded into the lipid bilayer to accept positively charged amino acids ⁹⁶. This phenomenon of positive charge acceptability at such membrane-associated domains was previously explained by a membrane orientation called “amino acid snorkeling”. Accordingly, positively charged lysine and arginine can integrate their uncharged side chains inside the lipid bilayer and place the positive charges near polar head

groups at the cytosol-membrane interface ⁹⁷. Thus, it was predicted that Fis1p could have similar membrane orientation that allows positive charges at certain portions of its TMD ⁹⁴.

2.3.3 Cellular function of Fis1p and its targeting at the MOM

Fis1p was first identified as a MOM TA protein, taking role in mitochondrial division as a dynamin-like enzyme in *S. cerevisiae*. On the other hand, another mitochondrial protein, Fzo1p, mediates mitochondrial fusion ⁹⁸. Thus, these two protein act in opposing fusion and fission events to maintain mitochondrial structure in yeast ⁹⁸. Fis1p is a conserved protein; it is also found in mammals, having a role in mitochondrial fission dependent processes ⁹⁹, although mitochondrial fission is not halted by *FIS1* deletion ^{100,101}, and FIS1 rather seems to function in mitophagy in mammalian cells ¹⁰². Deletion of Fis1p causes a typical networked phenotype in mitochondria, although mitochondrion still preserves its DNA and is capable of oxidative phosphorylation. On the other hand, loss of function in Fzo1p causes mtDNA loss upon disruption of mitochondria, and the cell becomes dependent on fermentation for ATP production ⁹⁸. However, double deletion of these two proteins balances the fusion and fission events, and mitochondrion is capable of oxidative phosphorylation as its wild-type structure is relatively preserved ¹⁰³. Interestingly, Fis1 was found also to operate fission of mammalian ⁸³ and yeast peroxisomes ¹⁰⁴. Thus, Fis1 is one of the few integral proteins capable of inserting both at peroxisomal ⁸³, mitochondrial ⁹⁸, and even ER membranes ⁷⁷.

Depletion of many of the TOM components does not seem to affect Fis1p insertion at the MOM. In fact its membrane insertion seems to be dependent on the presence of positively charged amino acids at the very C-terminus of the protein and on the ergosterol content of the target membrane ⁸⁵. In a yeast screen dedicated to uncover genes that might affect Fis1p targeting, no mutant strain in which insertion was completely blocked was identified. Instead, an ER-associated ATPase Spf1p, whose deletion partially shifted mitochondrial localization of Fis1p to ER, was reported to affect mitochondrial TA protein targeting. Consistent with the earlier observations regarding the lipid composition of the target membrane, *SPF1* deletion turned out to decrease cellular ergosterol levels, resulting in similar ergosterol content in mitochondrial an ER membranes, suggesting that the membrane composition is an important determinant of the organelle specificity of Fis1p ⁴⁸. The other key determinant seems to be the

positively charged amino acids following the TA domain as a mutant Fis1p lacking the positively charged amino acids was reported to localize at the ER along with the mitochondria⁹⁴. This ER localization was shown to be independent of the GET pathway, consistent with earlier studies⁷¹, suggesting that an alternative pathway might be playing a role for this targeting. Recently, a novel pathway alternative to SRP and GET pathways was reported to play a role in ER insertion of TA proteins, called SND pathway⁶⁸. It would be of great interest to see whether Fis1p or Fis1p TA mutants are following this alternative route. Lastly, it has been suggested that an ER to mitochondria protein transfer might be taking place through ER-mitochondria encounter site called ERMES^{76,77}, a membrane interaction facilitated by integral proteins found both at ER and mitochondrial membranes¹⁰⁵.

The relevancy of Fis1p with the evolution of mitochondrial protein import is the sequence characteristics of its TA that allows insertion at the MOM without requiring any of the known translocation machineries^{48,85}. It is possible that the early translocon that would drive the import of host-encoded proteins into proto-mitochondrion might have been inserted at the lipid bilayer with the help of a TA domain capable of self-insertion. TA sequences that resemble Fis1p TA are likely candidates to initiate the insertion of the first translocon during the course of eukaryogenesis, as Fis1p TA does not seem to depend on the assistance of any known import components^{48,85}. Such a TA sequence might have been derived from bacteria as bacterial TAs have previously been reported to localize to different intracellular structures resembling mitochondria, ER, peroxisomes, or vacuoles in *S. cerevisiae* cells¹⁰⁶. The sequence information of Fis1p could be a guide when searching for protein sequences that may have allowed membrane insertion of a progenitor MOM translocon predicted to evolve during eukaryogenesis. Also, Fis1 intrinsically functions in peroxisomes by inserting at peroxisomal membranes both in mammalian⁸³ and fungal systems^{104,107}. Although peroxisomes are not accepted as autonomous organelles, as they seem to import proteins through ER in fungi^{62,108}, they multiply by division and can also import proteins directly from the cytosol in both mammalian¹⁰⁹ and fungal systems¹¹⁰, unlike other members of the secretory pathway downstream of the ER¹¹¹. Thus, peroxisomes are also of great interest regarding their TA protein insertion mechanisms.

2.4 Protein biogenesis in peroxisomes

Peroxisomes are single-membraned organelles found in almost all nucleated cells. The metabolic pathways that peroxisomes mediate vary according to the type of the species or its environmental conditions ¹¹⁰. While peroxisomes are exclusively responsible for metabolism of fatty acids in yeast and many other organisms, the human peroxisomes complement mitochondrial fatty acid degradation, being specialized in β -oxidation of branched and very long chain fatty acids (VLCFA). On the other hand, common enzymes such as catalase that protects cells from oxidative damage by detoxifying hydrogen-peroxide are found in most of the peroxisomes ^{111,112}.

Peroxisomes import matrix and most of the membrane proteins through posttranslational import. All such peroxisomal proteins are nuclear encoded, synthesized on free polyribosomes, and can be posttranslationally imported to peroxisomes ¹¹³. Alternatively, some PMPs can localize to peroxisomal membranes following an ER to peroxisome route upon insertion at the ER membrane via cotranslational or posttranslational import mechanisms ¹¹⁰. Like other organellar translocation systems, peroxisomal matrix proteins contain targeting sequences that direct them to the membrane translocation site upon their recognition by relevant receptors. What makes peroxisomal import unique compared to mitochondria and chloroplast is its ability to import proteins in folded or even co-factor bound and oligomeric state ¹¹⁴. Peroxisomal membrane proteins (PMPs) on the other hand, seem to follow alternative pathways for peroxisomal import and these pathways are less understood compared to matrix protein import. Membrane peroxins (Table 2.1) encoded by PEX genes, cooperatively facilitate the import of matrix proteins through the membrane, and most of the PEX mutants are not competent for importing matrix proteins. Earlier studies suggest that loss of two of these peroxins in yeasts (Pex3p and Pex19p) and three in mammals (PEX3, PEX16, and PEX19) cause the organism to lack peroxisomal membrane structures ¹¹⁰, while more recent studies report evidence for peroxisomal ghosts containing some of the PMPs in *pex3Δ* or *pex19Δ* mutants ^{115–117}.

Multiplication of peroxisomes and the route of PMPs to their final destination on peroxisomal membranes have been a long-standing debate. Although peroxisomes were initially presumed as autonomous organelles like mitochondria and chloroplasts and never to form *de novo* ¹¹³, this view was challenged by the discovery that *pex3Δ* or *pex19Δ* mutants devoid of peroxisomal membrane structures can form peroxisomes *de novo* from the ER upon

re-expression of the deleted proteins ¹¹⁸. Recently involvement of mitochondria-derived PEX3 and PEX14 containing pre-peroxisomal vesicles that fuses with ER-derived PEX16 carrying vesicles to form mature peroxisomes has also been suggested in mammals ¹¹⁹. Whether peroxisomal proteins intrinsically follow an ER or mitochondria to peroxisome pathway, and there is a “continuous stream” of *de novo* peroxisome formation in wild-type cells is still argued ¹¹⁸.

2.4.1 Import of peroxisomal matrix proteins

Import of peroxisomal matrix proteins has been better understood compared to PMPs. Proteins destined for matrix space are post-translationally imported upon synthesis on free polyribosomes in the cytosol ¹¹³. Those proteins could be imported in fully folded state or as protein complexes passing through a series of steps on their way to matrix. Proteins are initially recognized by specific receptors found in the cytosol, and these receptor-cargo complexes reach to a docking complex on the peroxisomal membrane. Next, the cargo passes to the matrix space through a translocation pore, and the receptor is released from the cargo. The import cycle is completed by recycling of the receptor back to the cytosol ¹²⁰.

2.4.1.1 Signal recognition by receptors

Similar to most other subcellular protein import pathways, peroxisomal protein targeting is dependent on signal sequences called peroxisomal targeting signal (PTS) type 1 and type 2. PTS1 sequence is found at the C-terminus and used by most of the peroxisomal matrix proteins for membrane targeting. Firefly luciferase enzyme was the first protein discovered to contain this sequence ¹²¹. Mutations in the C-terminal segment of the luciferase protein prevented its peroxisomal transport. This finding suggested the presence of a tripeptide, known as SKL signal ¹²². Upon identification of different variations of this C-terminal tripeptide signal, PTS1 was described as an evolutionary conserved consensus sequence (S/A/C)-(K/R/H)-(L/M) ¹²³. In some cases, the C-terminal tripeptide does not seem to be sufficient for receptor binding, and amino acids adjacent to PTS1 provide additional interaction with the receptor ¹²⁴. The cytosolic receptor that recognizes PTS1 is the soluble protein Pex5p. Pex5p is a recognition-factor that

contains tetratricopeptide repeat domain on its C-terminus ¹²⁵. This domain interacts with the PTS1 by undergoing conformational change, and its N-terminus takes role in docking and recycling of the receptor ¹²⁶.

Unlike PTS1, the second targeting signal for peroxisomal matrix, PTS2, is closer to the N-terminus of the protein and found in a smaller subset of proteins. Similar to PTS1, PTS2 was first identified in an enzyme. Deletion analysis of the rat thiolase enzyme revealed that first 11 amino acids were sufficient for peroxisomal targeting and that the pre-sequence is cleaved off upon completing the protein import ^{127,128}. Protease DEG15 in plants ^{129,130} and TYSND1 in mammals ¹³¹ take a role in PTS2 cleavage while the cleavage does not take place in lower eukaryotes such as yeast ¹³⁰. In PTS2 sequences of different species, the conserved nonapeptide fits the consensus HR-(L/V/I/Q)-xx-(L/V/I/H)-(L/S/G/A)-x-(H/Q)-(L/A) ¹³². The cytosolic recognition-factor for PTS2 is the soluble protein Pex7p ¹³³. Interaction between the cargo protein and Pex7p receptor requires additional co-receptors that vary within different species. These co-receptors are Pex18p and Pex21p in *S. cerevisiae*, while in other fungi Pex20p has the homologous function. Such co-receptors are not found in mammals and plants. Instead, isoforms of Pex5p that cooperate with PEX7 for PTS2 recognition are considered as co-receptors in plants and mammals. Although these additional binding partners are non-orthologous, they share sequence similarities that serves as a common PTS2-receptor complex binding site ¹³⁴.

There are peroxisomal proteins do not contain neither of the two PTS signals and follow non-classical import mechanisms. When co-expressed with the native 3-ketoacyl-CoA thiolase, 50% of the truncated thiolase lacking its first 16 N-terminal amino acids localize to peroxisomes in *S. cerevisiae*. The truncated thiolase, which is restricted to cytoplasm when expressed alone, dimerizes with the full length thiolase, enabling its translocation to peroxisomes ¹³⁵. Such piggy-back import also exist in mammals. Cu/Zn superoxide dismutase 1 (SOD1) enzyme which do not contain an endogenous PTS, is imported into peroxisomes with the help of its interaction partner copper chaperone of SOD1 (CCS) that contains a PTS1 signal ¹³⁶. These protein complexes in different organisms can easily access to the lumen of peroxisomes due to the unique capacity of peroxisomes for importing complex protein structures. Finally, there are other peroxisomal proteins such as acyl-CoA oxidase (Pox1p) that reach peroxisomes without an interaction partner although lacking a PTS signal. Such proteins do bind to the recognition

protein Pex5p but this binding occurs through the N-terminus of Pex5p, instead of its PTS binding domain found on the C-terminus ¹³⁷.

2.4.1.2 Docking of receptor-cargo complex

Upon formation of the receptor-cargo complex in the cytosol, the complex associates with the peroxisomal membrane to initiate the cargo passage. This interaction requires the docking complex that is composed of Pex13p and Pex14p. In yeast, Pex17p completes the docking complex as the third protein ¹³⁸. The docking complex physically interacts with Pex5p or Pex7p receptors, allowing import of either of the PTS signals ¹³⁹. Pex13p is found integrated at the peroxisomal membrane exposing both its carboxyl and amino termini to the cytosol ¹⁴⁰. These cytosolic carboxyl and amino portions function as interaction sites with Pex5p ¹⁴¹ and Pex7p ¹⁴² respectively. Pex14p associates with Pex13p and functions as the binding unit of Pex17p in yeast ¹³⁹. Pex14 also interacts with both of the PTS receptors and in mammals, serves as the initial docking site for cargo-loaded PTS receptors before their translocation to the Pex13p containing complex ^{143,144}. In contrast, PTS2 receptor-cargo complex can dock at the peroxisomal membrane even in the absence of Pex14p in *S. cerevisiae*, suggesting that Pex13p is the initial docking site for PTS2 receptor in yeast ¹⁴⁵. Even though Pex17p is the third member of the docking complex in yeast, it is not found in more complex eukaryotes, and its contribution to the docking process is still to be revealed ¹⁴⁶.

In the later stages of peroxisomal import, docking complex also interacts with another protein complex called really interesting new gene (RING) finger complex that contains Pex2p, Pex10p, and Pex12p. This interaction allows PTS recycling back to the cytosol ¹⁴⁷, and details of this process are to be discussed in the following sections.

2.4.1.3 Cargo translocation and release

Upon docking of the cargo-receptor complex at the peroxisomal membrane, the cargo is translocated through the membrane and released into peroxisomal matrix, a process that allows import of enzymes in fully-folded or oligomeric state. Such a translocation process requires a unique translocon which is different from that of mitochondria or chloroplasts that can only

translocate unfolded proteins¹⁴⁸. This translocon should allow passage of protein complexes without changing their current conformation or disturbing the membrane and matrix environment of the peroxisome. Although the exact mechanism of this process is not clear, several plausible models have been suggested. Most of these models suggest the existence of an aqueous gate on the peroxisomal membrane. According to the most compatible model called ‘the transient pore model’, the import receptor triggers the formation of translocation pore that disassembles after completion of the translocation, while the receptors are recycled upon their ubiquitination¹⁴⁹. Indeed, although majority of PTS1 receptor Pex5p population is in a soluble state in the cytosol, a minor Pex5p fraction is found intact with the peroxisomal membrane showing all the characteristics of an intrinsic membrane protein and forming a stable complex with docking proteins. This observation suggests that Pex5p becomes integrated to the membrane as part of the translocation machinery upon loading its cargo, consistent with the ‘transient pore model’¹⁵⁰. In fact, presence of only the two peroxisomal proteins Pex5p and Pex14p is sufficient for the import of peroxisomal matrix protein Pex8p, supporting the role of Pex5p as a translocon¹⁵¹.

2.4.1.4 Release of receptors and their recycling

Once the cargo is imported into the peroxisomal matrix, the receptors are released back to the cytosol and ubiquitinated through a series of processes that requires ATP, unlike the importing steps that require no energy¹⁴³. The export is carried out by a complex containing two AAA-peroxins (ATPases associated with diverse cellular activities), Pex1p, and Pex6p¹⁵². These two peroxins are partially cytosolic, and they localize to peroxisomes with the help of Pex15p in yeast and PEX26 in mammals, two homologous proteins that function as the binding site for Pex6p to recruit Pex1p^{153,154}. AAA-peroxin complex supplies the energy necessary for releasing the receptor to the cytosol, a mechanism not fully understood but known to be dependent on receptor ubiquitination¹⁵⁵.

In yeast, PTS1 receptor Pex5p ubiquitination is carried out by Pex4p that is found on the cytosolic face of the peroxisomal membrane¹⁵⁶ and by ubiquitin conjugating proteins in mammals¹⁵⁷. Although the exact mechanism of Pex1/Pex6 binding with ubiquitinated Pex5p is unknown, discovery of a novel Pex6p binding partner, ubiquitin-binding adaptor protein AWP1, suggests that it might play a role in Pex5p recognition by Pex1/Pex6 complex¹⁵⁸.

PTS2 receptor Pex7p export also requires its ubiquitination, a process that is not fully understood but is known to be Pex4p and the RING complex dependent ¹⁵⁹.

After the receptor export to the cytosol is completed, the ubiquitin is removed so that the receptor could start a new cycle. Cleavage of the ubiquitin is carried out by deubiquitin hydrolases, also named deubiquitinating enzymes (DUBs), found in the cytosol ¹⁶⁰.

2.4.2 Import of peroxisomal membrane proteins

The import pathways for peroxisomal membrane proteins PMPs differs from matrix-localized peroxisomal proteins. Defects in peroxisomal membrane proteins cause mislocalization of matrix enzymes to the cytosol, and PMPs are found on peroxisomal membranes called membrane ghosts ¹⁶¹. These peroxisomal membrane ghosts are found in almost all of the PMP mutants although absence of Pex3p and Pex19p in yeast (also PEX16 in mammals) (Table 2.1) were suggested to be peroxisomal ghost deficient in earlier studies ¹⁶². However, there are several studies in yeast that argues otherwise ^{116,117}. According to one of these works, fluorescently tagged Pex14p can localize to vesicular structures that are adjacent to ER in *pex3Δ S. cerevisiae* cells, suggesting that those membrane structures could be pre-peroxisomal vesicles (PPVs) that would mature into normal peroxisomes upon Pex3p reintroduction ¹¹⁶. In a more recent study, localization of red fluorescently tagged Pex14p was observed compared to 19 other GFP tagged PMPs in *pex3Δ S. cerevisiae*, and it was represented that Pex14p containing structures colocalized with several other PMPs including Pex13p, Pex17p, and Pex5p ¹¹⁷. Thus, a combined model, in which two pathways operate for the insertion of PMPs at the peroxisomal membrane, has been suggested for yeast. In yeast, most of the PMPs can insert directly at the already existing peroxisomes, upon their interaction with the Pex19p receptor/chaperone followed by docking of the receptor cargo complex on the Pex3p at the peroxisomal membrane ¹⁶³. On the other hand, at least a subpopulation of the PMPs follow an ER to peroxisome pathway, a process that contribute to the formation of new peroxisomes even if the cell lacks a pre-existing peroxisome population ¹⁰⁸. While PEX16 is also required as a cytosolic factor for PMP import at already existing peroxisomes ¹⁶³, the existence of the ER route for most of the PMPs in mammalian cells is still argued ^{164,165}.

2.4.2.1 Direct import of PMPs

Direct import at peroxisomes involves posttranslational targeting of PMPs to the peroxisomal membrane, upon their synthesis on free polyribosomes in the cytosol. Most of these proteins are class 1 PMPs and their import follows a Pex19p dependent pathway. In this pathway, Pex19p is suggested to function as a soluble receptor or as a chaperone. Recognition by Pex19p is a prerequisite for class 1 PMP import ¹⁶⁶. Pex19p then recruits its cargo to membrane bound Pex3p ¹⁶⁷. By binding to newly synthesized PMPs, human PEX19 maintains their stability and solubility in the cytosol along with cytosolic PEX16 ¹⁶⁸, while Pex19p is the only known cytosolic factor mediating the direct pathway in yeast cells ¹⁶². In this context, overexpression of Pex19p in *pex3Δ* cells that lack mature peroxisomes stabilizes PMPs in the cytosol, preventing their degradation ¹⁶⁹. However, Pex19p on its own is not sufficient for insertion; the interaction between Pex19p and Pex3p is also needed ¹⁷⁰. The relevance of this interaction is characterized by studies with mutant cells that cannot assist Pex3p-Pex19p interaction, preventing insertion of PMPs into peroxisomes ¹⁷¹. Finally, Pex19p is also suggested to function in indirect pathway by playing role in Pex3p import from ER to peroxisomal membranes ¹⁷².

Pex3p is an integral membrane protein anchored to the peroxisomal membrane through its N- terminus while rest of the protein is forming ‘twisted six-helix bundle’ in the cytosol ¹⁷³. The hydrophobic apex of the α -helical structure provides the binding site for Pex19p amphipathic helix ¹⁷⁴ while the other hydrophobic side at the base of the protein close to the lipid bilayer maintains its integrity on the membrane ¹⁷⁵. This topology of Pex3p on the membrane surface allows it to function as a docking factor on the peroxisomal membrane for Pex19p-PMP complex and facilitate import of PMPs. Recent studies in yeast also suggested that Pex3p might function as a receptor for peroxisome retention factor Inp1p that controls peroxisome division and causes uncharacteristic chaotic movement of peroxisomes when deleted ¹⁷⁶.

2.4.2.2 Indirect import of PMPs and involvement of the ER

Indirect import of PMPs to peroxisomal membrane involves ER, an organelle whose role in peroxisomal biogenesis has long been debated. Although for a long time peroxisomes were accepted as autonomous organelles like mitochondria and plastids, electron microscopic images in 1972 showing ER and peroxisomes in close association suggested that peroxisomal membranes are sourced from ER ¹⁷⁷. On the other hand, studies revealing posttranslational import of matrix and PMPs to pre-existing peroxisomes upon their synthesis from cytosolic polyribosomes ¹⁷⁸ was more consistent with the model that peroxisomes are autonomous organelles ¹¹³. However, later findings were not in support of this view. Involvement of the ER in peroxisomal biogenesis was most prominently represented in a study with *pex3Δ* and *pex19Δ* cells ¹⁷². Although these cells are devoid of mature peroxisomes in the absence of either of the two proteins ^{162,179,180}, reintroduction of these proteins allowed formation of mature peroxisomes ¹⁷². However, the question of whether this *de novo* peroxisome formation from the ER happens only in the absence of mature peroxisomes or there is a continuous flow of peroxisomal membranes from the ER remained unanswered. Later, it was indicated that there are several PMPs routed to peroxisomes through an ER directed pathway. In this study, peroxisomal membrane proteins Pex2p and Pex16p were reported to be N-glycosylated, a process that is specific to ER, in the yeast *Yarrowia lipolytica*, indicating that some of the PMPs are routed to peroxisomes via ER. This ER targeting of Pex2p and Pex16p was also reported to be affected by mutations in genes *SEC238* and *SRP54* although the exact role of these proteins were not specified at the time ¹⁸¹. Later, several other PMPs including Pex3p, Pex22p, Pex15p, and Pex26p were represented to be trafficked to peroxisomes via ER in different species ¹¹¹. Among these different PMPs, the targeting of class 2 PMP Pex3p to peroxisomal membranes is the most interesting route as Pex3p itself is a docking factor for other PMPs, mediating their membrane insertion together with the cytosolic receptor Pex19 that recruits them on Pex3p ¹⁶⁷.

Pex3p differs from most other PMPs that contain PTS1, as it is a class 2 type of PMP containing PTS2. This class of PMPs including Pex3p ¹⁸², Pex16p ¹⁸³, and Pex22p ¹⁸⁴ do not require Pex19p for peroxisomal membrane insertion. Different studies indicated evidence for Pex3p peroxisome targeting to occur through the ER. In one of these studies, the trafficking of fluorescently tagged Pex3p upon its expression in *pex3Δ S. cerevisiae* was observed. These real-time microscopic images suggested that fluorescently tagged Pex3p initially concentrates on ER foci and later buds off to mature into functional peroxisomes ¹⁷². Later, import of human PEX3 to the ER membrane and its subsequent exist in pre-peroxisomal vesicles was also shown

in mammalian cells ¹⁸⁵. Although it is accepted that PTS2 containing PMPs including Pex3p do not require Pex19p interaction for peroxisomal import ¹⁸², Pex19p still might be needed for the formation of Pex3p containing mature peroxisomes as budding off the Pex3p concentrated ER foci was shown to be Pex19p dependent ¹⁷². More interestingly, when Pex3p is directed to ER with an ER targeting signal in yeast *S. cerevisiae*, it restores the *pex3Δ* phenotype of the yeast, the ER targeting signal is successfully cleaved, and mature Pex3p ends up in peroxisomes ¹⁸⁶. The literature suggests that the ER might not be the only organelle contributing in *de novo* peroxisome formation and/or PMP targeting. Pex3p targeted to mitochondria was reported to induce *de novo* peroxisome formation in peroxisome deficient *pex3Δ* yeast cells ¹⁸⁷. Consistent with this work, pre-peroxisomal vesicles harboring peroxisomal import receptors PEX3 and PEX14 were reported to release from mitochondria, fuse with ER-derived PEX16 carrying structures, and mature into import competent peroxisomes in mammalian cells ¹¹⁹.

The machinery that allows Pex3p import to ER was reported to be dependent on the ER channel protein Sec61p. In *S. cerevisiae*, *sec61-2* mutant strain expressing temperature sensitive Sec61p, peroxisomal accumulation of Pex3p upon its induced expression was reported to be significantly reduced ¹⁸⁸. ER proteins are routed to ER Sec61p translocon through co-translational SRP dependent import ⁵⁷ or through posttranslational import, dependent on cytosolic heat-shock protein chaperones and Sec63 complex that recognizes the signal sequence ⁶⁴. Although many other PMPs including Pex8p, Pex13p, and Pex14p were observed not to integrate into the ER membrane but to remain in the cytosol upon depletion of Sec63 complex components Sec62p and Sec63p, it did not help to distinguish between the two pathways ⁶² as Sec63p also functions in SRP-dependent co-translational insertion ¹⁸⁹. Later, the same group suggested that different PMPs (docking and RING finger groups) sort into distinct vesicles on ER subdomains and subsequently fuse to form mature peroxisomes in a Pex1p and Pex6p dependent manner ¹⁰⁸. More recently, another ER targeting route constituted by three uncharacterized proteins named Snd1p, Snd2p, and Snd3p (for SRP-independent targeting) was suggested to be alternating for TA protein targeting pathways in yeast ⁶⁸. It remains to be discovered whether this alternative pathway is also involved in PMP targeting to peroxisomes.

2.4.2.3 Targeting of tail-anchored PMPs to peroxisomes

Pex15p that functions in receptor recycle in yeast peroxisomes and its mammalian orthologue PEX26⁹³, are two homologous PMPs containing a tail anchor. Although the two proteins share no sequence similarity except for the peroxisomal targeting sequence⁹³, they both function in recruiting PEX6 to peroxisomal membrane, to initiate receptor release to the cytosol during receptor recycling¹⁵³.

Pex15p, like most other PMPs is an essential protein for the formation of mature peroxisomes. In *pex15Δ* yeast, matrix enzymes such as thiolase or catalase were reported to be exclusively cytosolic, while peroxisomal membrane protein Pex13p was membrane bound suggesting the presence of import incompetent peroxisomal vesicles called peroxisomal ghosts⁸⁴. Overexpression of Pex15p, similar to many other TA proteins, causes its mislocalization to other organelles such as ER⁸⁴. The last 30 amino acids after the TMD of Pex15p is necessary and sufficient for its peroxisome specific targeting, as bulk of the Pex15pΔ30 was found to be associated with ER membranes while deleting only the last 12 amino acids did not affect its peroxisomal targeting⁸⁴. Interestingly, peroxisomal targeting information is evolutionarily conserved between fungal and mammalian systems. When mammalian orthologue PEX26 was expressed in *S. cerevisiae*, it extensively localized to yeast peroxisomes. Similarly, Pex15p expressed in mammalian cells localized to mammalian peroxisomes although neither of the two proteins could complement for the loss of their orthologue protein when expressed in the respective organism⁹³.

Pex15p seems to be a type of TA protein that follows the secretory pathway, at least as an alternative route. In a yeast screen aiming to identify Get3p-interacting TA proteins, Pex15p was found to be interacting with the GET complex similar to other TA proteins destined for the secretory system such as the SNAREs Tlg2p and Sec22p. On the other hand, Fis1p, which can directly insert at the MOM, did not interact with the GET machinery. Moreover, overexpression of Pex15p in *get3Δ* or *get1/2Δ* mutants, causes its mislocalization to mitochondria, a phenotype that was not observed in wild-type yeast, suggesting that saturation of GET complex could result in Pex15p mistargeting to mitochondrial membranes⁷¹. Later, real-time pulse-chase experiments made with *get3Δ* yeast further supported the view that Pex15p insertion on peroxisomal membranes is dependent on GET pathway. Accordingly, upon induced expression of fluorescently tagged Pex15p, the protein was observed to reach peroxisomes in wild-type strains while in *get3Δ* mutants, Pex15p was mostly not detectable and never reached peroxisomes⁶². Interestingly, this mitochondria-localized phenotype of Pex15p was also

observed in other yeast mutants. Upon deletion of Msp1p, an ATPase found at the MOM, Pex15p displayed dual localization on mitochondria and peroxisomes. Furthermore, Pex15p localization was exclusively mitochondrial in *get3Δ msp1Δ* double-mutant cells supporting the notion that Pex15p is constitutively mis-targeted to mitochondria in wild-type cells, and Msp1p acts downstream of GET pathway to extract and degrade Pex15p mis-targeted to mitochondria¹⁹⁰.

Pex15p also seems to reach peroxisomes through a machinery independent of the GET pathway. Unlike *pex15Δ* cells that are devoid of peroxisomes, *get3Δ* or *get1/2Δ* cells do have peroxisomes^{62,71}. As Pex15p was shown to associate with Pex19p, it is possible that Pex15p is targeted to peroxisomes via direct pathway as well, upon interacting with Pex19p¹⁹¹. On the other hand, unlike its yeast orthologue, PEX26 TA PMP is inserted to peroxisomal membrane independent of the GET/TRC machinery¹⁹². In fact, targeting of PEX26 to peroxisomes heavily depends on PEX19-PEX26 interaction, suggesting that direct pathway operated by PEX3/PEX19 is followed by PEX26 on its way to peroxisomes⁸⁶. Moreover, human Fis1, a TA protein that dual localizes to mitochondria and peroxisomes⁸³, was also reported to be dependent on PEX19 specifically for peroxisomal targeting, although such a requirement was not suggested for its mitochondrial localization¹⁹³.

As shown by many of these studies, there is no single machinery for tail-anchored PMPs even within the same organism. Yeast Pex15p seems to follow both the direct targeting route upon Pex19p interaction¹⁹¹ and the indirect targeting route via the ER through the GET pathway⁶², while mammalian PEX26 is exclusively dependent on the direct targeting machinery^{86,192}. As bacterial TAs were shown to localize to different intracellular structures resembling mitochondria, ER, peroxisomes, or vacuoles in *S. cerevisiae* cells¹⁰⁶, a peroxisome localized bacterial TA might be used as a functional tool to study the complex protein targeting and membrane maturation of peroxisomes. Although there are peroxisome-localized fluorescently tagged fusion proteins that allow organelle visualization *in vitro*, they either mislocalize to other organelles due to the overexpression of the protein¹⁹⁴, or the fluorescent tag that they possess interfere with peroxisomal morphology, number, and distribution because of the intrinsic role of the protein in the organelle¹⁶⁹. Thus, a bacterial TA, the sole purpose of which is to insert at peroxisomal membranes, having no native function in the organelle, might allow peroxisomal visualization with reduced artifacts.

Table 2.1 Peroxins involved in peroxisomal protein import in *S. cerevisiae* cells along with their human orthologues ¹¹⁰.

<i>S. cerevisiae</i>	<i>Homo sapiens</i>	Process	Complex
Pex1	Pex1	Export of receptors	AAA+ complex
Pex2	Pex2	Ubiquitination of receptors	RING ubiquitin ligase complex
Pex3	Pex3	PMP docking site	Pex3/Pex19/cargo complex
Pex4	UbcH5a/UbcH5b/UbcH5c	Ubiquitination of receptors	Ubiquitin conjugation complex
Pex5	Pex5S/Pex5L	Cargo targeting and translocation	PTS1 receptor, import pore
Pex6	Pex6	Export of receptors	AAA+ complex
Pex7	Pex7	Targeting	PTS2 receptor
Pex8		Translocation and cargo release	Intraperoxisomal peroxin
Pex10	Pex10	Ubiquitination of receptors	RING ubiquitin ligase complex
Pex12	Pex12	Ubiquitination of receptors	RING ubiquitin ligase complex
Pex13	Pex13	Docking	Docking complex
Pex14	Pex14	Docking and translocation	Docking complex and import pore
Pex15	Pex26	Export of receptors	AAA+ complex
	Pex16	PMP docking site	Pex3/Pex19/Pex16/cargo complex
Pex17		Docking	Docking complex
Pex18/Pex21	Pex5L	Targeting	PTS2 coreceptor

Pex19	Pex19	PMP targeting	PMP receptor
Pex22	Pex22	Ubiquitination of receptors	Ubiquitin conjugation complex



Chapter 3

BACTERIAL TAIL ANCHORS CAN TARGET TO THE MITOCHONDRIAL OUTER MEMBRANE

3.1 Introduction

Endosymbiotic theories for eukaryote origin suggest that the eukaryotic organelle mitochondria was once a free living organism itself¹³. As the early atmosphere of the world was anaerobic, the first organisms were anaerobic bacteria that produce ATP via metabolic pathways that probably resembled what we call fermentation¹⁹⁵. These anaerobic organisms later evolved into photosynthetic bacteria, resulting in the formation of aerobic atmosphere. Presence of oxygen, in turn, allowed development of an aerobic metabolism in prokaryotes¹³. According to the endosymbiotic theory, an aerobic bacterium was integrated into a proto-eukaryotic host through a symbiotic association. After this association, they eventually refashion into an altered organism through permanent incorporation of the endosymbiont into the host, and transformation of the endosymbiont into a distinct organelle, mitochondria¹⁹⁶. It is favored that after this event, the endosymbiont and its host evolved into the last eukaryotic common ancestor (LECA), an organism that is assumed to possess most of the derived features of present-day eukaryotes¹⁹⁷. After the LECA, several eukaryotic supergroups have emerged^{198,199} including Excavata, Amoebozoa, SAR, Archaeplastida, which contains red algae and green plants²⁰⁰, and Opisthokonta that includes Metazoa, Fungi, and related unicellular lineages²⁰¹, although the order and diverging point of each supergroup is still debated.

In the course of eukaryogenesis, mitochondrion, no longer being a free-living organism, decreased its genomic size and transferred most of its protein encoding genes to the host

nucleus, a process called endosymbiotic gene transfer (EGT) ³². Whereas some of the endosymbiotic genes were completely lost as their products were either substituted by a nuclear homolog or lost functionality during the course of evolution ³¹. According to one theory not only its genetic material formed some part of the host genome, but also its outer membrane itself might have formed the endomembrane system ²⁰². Already possessing mitochondrial targeting information for many of its proteins ²⁰³, most of the protein products of these genes easily acquired new functions within the eukaryotic endomembrane system ⁵. On the other hand, the protein products of some of the genes transferred to the nucleus were still needed in the mitochondria. The product of these nuclear encoded genes lost through EGT along with the gene products acquired through (horizontal gene transfer) HGT from other organisms or that were evolved during eukaryotic evolution needed to target mitochondria ⁵. This requires protein recognition in the cytosol, translocation through the mitochondrial membranes, and relocation to the matrix space or assembly within the membranes, a series of conserved events that take place in modern eukaryotes ³². Although targeting information to the mitochondria was suggested to have been already existed within the endosymbiont ²⁰³, no counterparts of the mitochondrial translocating machinery have been detected in prokaryotes ³³. Thus, formation of an import machinery was the only hurdle to re-target such proteins to mitochondria.

Origins of the mitochondrial import machinery have long been debated. As there is no evidence for bacterial protein import machineries to be the origins of the mitochondrial translocator proteins, it is possible that modern mitochondrial translocons have evolved *de novo* ³³. The translocon found at the mitochondrial outer membrane (MOM) that allows import of mitochondrial matrix and membrane proteins is the TOM complex, a protein that is very much conserved among different species ³⁸. Although it is the main gate allowing protein import into mitochondria, the insertion of the core components of this translocon takes place through the TOM complex itself. Tom40 is the core component of the TOM complex³⁸. It is the pore forming subunit, constituting the hydrophilic channel that allows import of the preproteins ³⁹. However, this protein seems to require pre-existing TOM complexes for assembling at the MOM ⁹. Thus, integration of the first translocon at the MOM remains as an evolutionary puzzle.

Formation of an initial gate at the proto-mitochondrial outer membrane was probably an important step for the evolution of the remaining translocator components. It has been

suggested that the three components of the TOM complex, Tom40, Tom22, and Tom7 proteins were developed within the last eukaryotic common ancestor (LECA)³³. Existence of Tom7 within the LECA became rather questionable¹⁹⁸, with the discovery of an analogous translocase complex named ATOM within the kinetoplastid mitochondrial outer membrane, a complex that lacks a Tom7 counterpart^{204,205}. As a homologous Tom7 sequence has not yet been detected in Excavata, including *Naegleria gruberi*²⁰⁴ and kinetoplastids such as *Trypanosoma brucei*^{205,206}, two models for the origin of Tom7 have been suggested. According to the first model, Tom7 was lost from the early TOM complex possessing Tom40, Tom22, and Tom7 in divergence to kinetoplastids. Alternatively, after the divergence of Excavata, Tom7 was integrated into the TOM complex that contained Tom40 and Tom22¹⁹⁸. However, as Excavata was suggested not to be an early branch within the phylogenetic tree¹⁹⁹, the first model that suggests the presence of Tom40, Tom22, and Tom7 in the early TOM complex was favored¹⁹⁸. On the other hand, receptors that recognize mitochondrial presequences evolved convergently. Counterparts of the receptor Tom20 were acquired independently within the eukaryotic tree^{205,207}. Then how could such a primitive TOM complex containing Tom40, Tom22, and Tom7 components but not the necessary receptors for protein recognition mediate protein import into mitochondria? It is predicted that the interaction between the positively charged residues within the presequence and the acidic patches found at the TOM complex allowed the primitive protein import¹⁹⁸, a process that has been previously proposed as the acid chain model²⁰⁸. In the case of the inner membrane translocon TIM the only homologue protein possessed by kinetoplastids is Tim17²⁰⁹. However, three components of the complex, Tim17, Tim22, and Tim23, are predicted to be present within the LECA^{198,210}. Likewise, the model for the evolution of the early TIM complex also suggests that two of these proteins, Tim22 and Tim23, were lost during divergence to kinetoplastids²¹⁰. Interestingly, the last ancestor of the TIM23 complex was predicted to possess all of its known components, Tim23, Tim17, Tim50, and Tim21¹⁹⁸, identical to components found in yeast³³. The early TIM22 complex was also suggested to contain all of the core components, Tim22 and small Tims. Probably, TIM complex also had an acidic feature at the LECA so that precursor proteins are sorted cooperatively by TOM and TIM complexes¹⁹⁸. PAM complex components Tim44, Pam 16, and, Pam18 were also suggested to be present at the LECA¹⁹⁸. In fact Tim44, which tethers PAM and TIM23 complexes, has even bacterial origins²¹¹. Finally, the timing for the emergence of other

components such as Tom5 or Tom6 remains to be discovered ¹⁹⁸. All of these evolutionary steps were obviously initiated by the integration of an early translocon at the proto-mitochondrial outer membrane.

There are several theories for the integration of the first mitochondrial outer membrane translocon that led to the evolution of other components and eventually the modern translocating machinery. According to one scenario, the early translocon that eventually evolved into the current translocation machinery should have integrated at the MOM through a self-inserting mechanism ⁴⁰. However, none of the known translocons found in the eukaryotic ^{9,43,45} or prokaryotic membranes ⁴¹ have the self-inserting ability. YidC, the homolog of mitochondrial inner membrane translocating protein Oxa1p, inserts at bacterial inner membrane through pre-existing YidC ^{42,44}. Similarly, The eukaryotic homolog of YidC, Oxa1p requires pre-integrated Oxa1p ⁴³ or TIM22 requires pre-integrated TIM22 ⁴⁵. They are found integrated on the relevant lipid bilayer as they are inherited to the daughter cells by the division of pre-existing organelles or from the membranes of the mother cell ⁴⁰. Thus, it is possible that the early translocon of the proto-mitochondria may have evolved through unassisted insertion ⁴⁰.

Even though translocator proteins cannot self-insert at biological membranes, there are other type of membrane proteins capable of unassisted insertion. Antimicrobial polypeptides produced by prokaryotes ⁵⁴ and eukaryotes ⁵¹ are able to spontaneously insert at lipid bilayers. Moreover, certain the mitochondrial proteins are also capable of spontaneous insertion at the MOM, including some of the pro-apoptotic proteins that can initiate pore formation at the MOM ⁷⁹. All such proteins contain a similar hydrophobic amino acid patch close to the C-terminus called tail anchor (TA) that is necessary for their spontaneous insertion ⁵⁶. Fis1p, the conserved MOM protein that is responsible for mitochondrial division events, is also tail-anchored. Current evidence suggests that Fis1p insertion at the yeast MOM is not dependent on the TOM complex ⁸⁵. In fact, its membrane insertion does not seem to depend on any of the cellular factors in yeast, but the ergosterol content of the target membrane ⁴⁸. It is one of the exceptional integral membrane proteins that can intrinsically insert and function at distinct organelles. It not only operates mitochondrial fission but also has a similar function at peroxisomal division ⁶⁰.

Here, we hypothesized that tail-anchored proteins that may have played a role in the integration of an initial translocon at the MOM would likely have been obtained from

prokaryotes. To follow the intracellular fate of bacterial TAs in eukaryotic cells, we expressed bacterial TAs generated from proteobacterium *Escherichia coli* in the yeast *Saccharomyces cerevisiae*. Indeed, some of the bacterial TAs displayed insertion and functionality at mitochondrial membranes when Fis1p TA is replaced with these TAs. These results enabled us to propose a scenario for the evolution of mitochondrial translocons.

3.2 Results

3.2.1 Bacterial TAs can insert at eukaryotic membranes

Previous studies suggest that the TA of Fis1p is necessary⁹⁸ and sufficient⁸⁵ for Fis1p to appropriately insert at the MOM. It has been predicted that Fis1p has a final MOM topology that its positively charged C-terminus protrudes to the intermembrane space of mitochondria, the amino-terminal bulk of the protein faces the cytosol, and these two hydrophilic domains are connected through a hydrophobic domain called transmembrane domain (TMD) integrated to the MOM⁵⁶.

In search of prokaryotic TAs, which could insert at eukaryotic membranes, we took advantage of previous data generated in our lab¹⁰⁶. In this work, *E. coli* polypeptides with a single predicted TMD, containing 15 or less amino acids carboxyl-terminal to the TMD and more than 30 amino acids amino-terminal to the TMD were analyzed for their intracellular localization in the yeast *S. cerevisiae*. Out of the eight bacterial TAs tested in this work, we selected four (Figure 3.1) that were reported to localize to mitochondria or peroxisome-like structures in yeast cells¹⁰⁶. In order to assess whether these bacterial TAs can allow insertion of Fis1p when the TA of Fis1p is replaced with any of these TAs, we used an assay previously developed in our laboratory⁹⁴. According to this assay, Gal4 transcription factor is fused to a protein, intracellular location of which is predicted to be a membrane outside of the nucleus. If this fusion protein fails to insert at its target membrane, it would localize to nucleus and activate Gal4-responsive promoters that allow proliferation under selective media. In other words, if yeast cells expressing this fusion protein fail to survive in selective medium, it would suggest that the fusion protein is inserted at its target membrane. Similar to previous assays, while native Fis1p in Gal4-sfGFP-Fis1 protein construct did not cause a

proliferation defect in non-selective medium (SC-Trp), *S. cerevisiae* cells expressing the same construct failed to survive in selective medium (SMM-His +20 mM 3-AT) that requires the activation of Gal4p-driven *HIS3* gene (Figure 3.2). On the other hand, fusion proteins carrying Fis1p without its TA or with a A144D charge substitution within the TA, which was previously shown to prevent insertion at the MOM⁹⁴, activated Gal4-dependent *HIS3* gene to allow proliferation at selective medium. To test bacterial TAs in the same assay, we used the TAs of bacterial proteins ElaB, YqjD, and TcdA that were shown to localize to mitochondria-like structures and that of bacterial protein YgiM that was reported to enrich at structures that resemble peroxisomes¹⁰⁶. When the TA of Fis1p is replaced with any of these four TAs, yeast cells did not survive on medium requiring *HIS3* gene activation, suggesting their insertion at an intracellular membrane outside the nucleus, consistent with the earlier observations¹⁰⁶, or degradation of the Gal4-fusion protein.

P0ADT8	YgiM	<u>IIMQWFMYG</u> GGVLGLGLLLGLVLP <u>HLIPSRKRKDRWMN</u> ⁺ ^{+++++ - +}
Q46927	TcdA	<u>GFGAATMVTATFGFVA</u> VSHALKKMMAKAARQG ⁺⁺ ⁺ ⁺
P0AEH5	ElaB	<u>PWQGIGVGA</u> AVGLVLGLLLARR ⁺⁺
P64581	YqjD	<u>WTGVGIGA</u> AIGVVLGVLLSRR ⁺⁺
P40515	Fis1	<u>VVVAGGVLAGAVAVAS</u> FFLRNKRR ⁺ ⁺⁺⁺

Figure 3.1 A list of predicted TAs investigated in this study. The UniProt accession numbers and names of selected proteins that the TAs are belonged to are provided, along with the sequences of the predicted TAs. The single predicted membrane-passing domain is shown by a purple line. Charged amino acids are also indicated. The relevant portion of the *S. cerevisiae* Fis1p TA is also shown for sequence comparison.

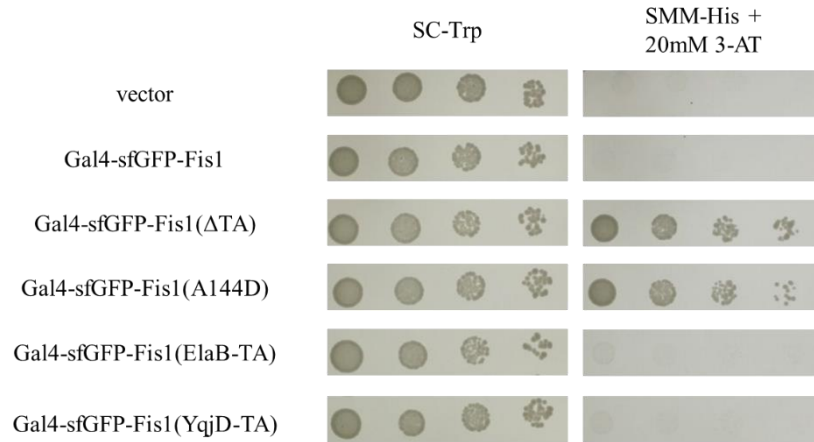


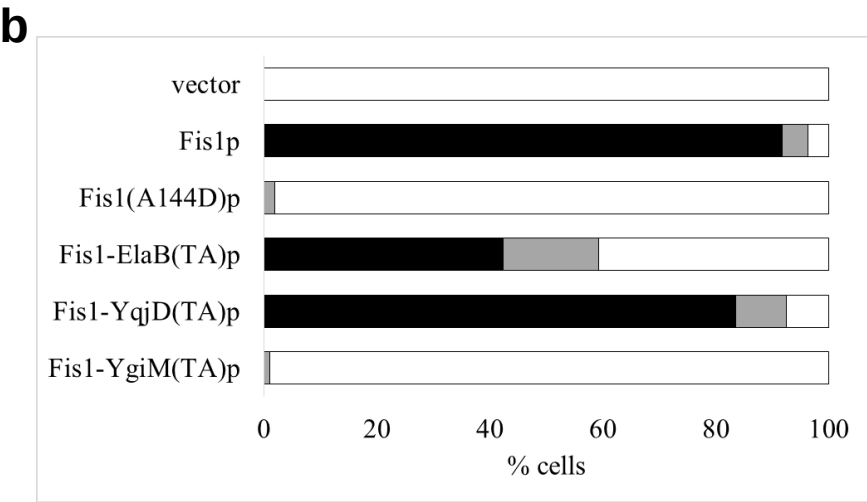
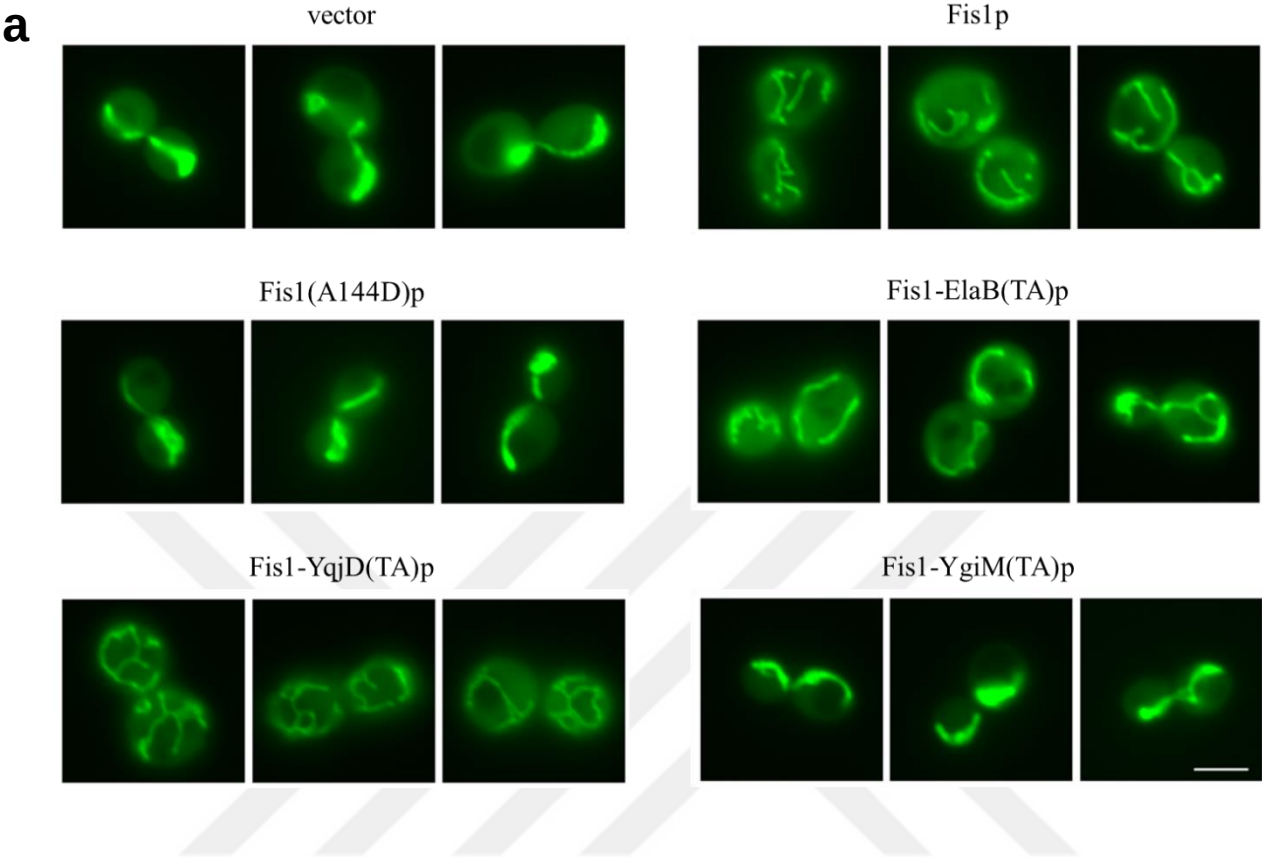
Figure 3.2 A proliferation-based assay suggests the membrane insertion of ElaB and YqjD TAs. Strain MaV203, containing a Gal4-driven *HIS3* gene, was transformed with plasmids expressing Gal4-sfGFP-Fis1p (b100), a variant lacking the Fis1p TA (b101), a mutant containing the A144D mutation in its TA (b180), or the Gal4-sfGFP-Fis1p construct with the Fis1p TA replaced with that of either ElaB (b313) or YqjD (b314). The same strain was also transformed with empty vector pKS1 as control. Transformants were cultured in SC-Trp medium, then, following serial dilution, spotted to SC-Trp or SMM-His +20 mM 3-AT and incubated for 2 d.

3.2.2 Bacterial TAs are functional at the MOM

To test whether these two bacterial TAs ElaB and YqjD would also allow proper insertion at the MOM, we aimed to study the functionality of these TAs when they replace the TA of Fis1p. As Fis1p drives mitochondrial division in *S. cerevisiae*, mutant cells lacking this protein have a typical interconnected mitochondrial phenotype because of ongoing fusion events^{98,103}. To observe the mitochondrial distribution, we expressed different Fis1p constructs, in *fis1Δ* *S. cerevisiae* cells expressing mitochondria-localized GFP. As expected, expression of wild-type Fis1p restored normal mitochondrial phenotype, while Fis1p containing the charge substitution A144D or lacking the entire TA did not change the interconnected mitochondrial morphology (Figure 3.3a and b). Interestingly, replacement of Fis1p TA with bacterial ElaB or YqjD TAs allowed restoration of wild-type mitochondrial morphology by promoting mitochondrial division, suggesting their functional integration at the MOM. On the other hand, a control bacterial TA of YgiM that is not trafficked to

mitochondria¹⁰⁶ could not restore mitochondrial morphology and distribution, when Fis1p TA is replaced with YgiM TA.

In search of further evidence for the functionality of Fis1p having its native TA replaced with that of ElaB or YqjD bacterial proteins, we used another assay based on mitochondrial fission and fusion events that would allow cell proliferation⁹⁴. Fis1p functions in mitochondrial division events while another mitochondrial protein, Fzo1p, mediates mitochondrial fusion. Ongoing mitochondrial division driven by Fis1p in the absence of Fzo1p leads to mitochondrial fragmentation and loss of mitochondrial DNA. *S. cerevisiae* cells lacking mtDNA cannot survive in non-fermentable medium^{98,103}, and this outcome can be used in an assay to test Fis1p functionality⁹⁴. Consistent with the previous studies, *fzo1Δ fis1Δ* yeast cells expressing wild-type Fis1p could not survive in non-fermentable medium because of ongoing mitochondrial fission driven by the functional Fis1p in the absence of Fzo1p. Whereas, expression of the non-functional Fis1(A144D) variant did not change the respiration capacity of yeast cells with the same genetic background (Figure 3.3c). Expression of Fis1p having its native TA replaced by that of bacterial TAs ElaB or YqjD, provided sufficient mitochondrial fission that resulted in mtDNA loss in *fzo1Δ fis1Δ* cells, indicating functional ElaB and YqjD TA insertion at the MOM. Surprisingly, Fis1-TcdA(TA) protein that did not show enough functionality to restore mitochondrial morphology, prevented survival in non-fermentable medium when expressed in *fzo1Δ fis1Δ* cells, suggesting functionality to some extent (Figure 3.4). Consistent with the earlier work¹⁰⁶, such limited functionality was not observed upon Fis1-YgiM(TA) expression in the same genetic background, suggesting its localization at a different membrane structure or degradation of the protein (Figure 3.3c).



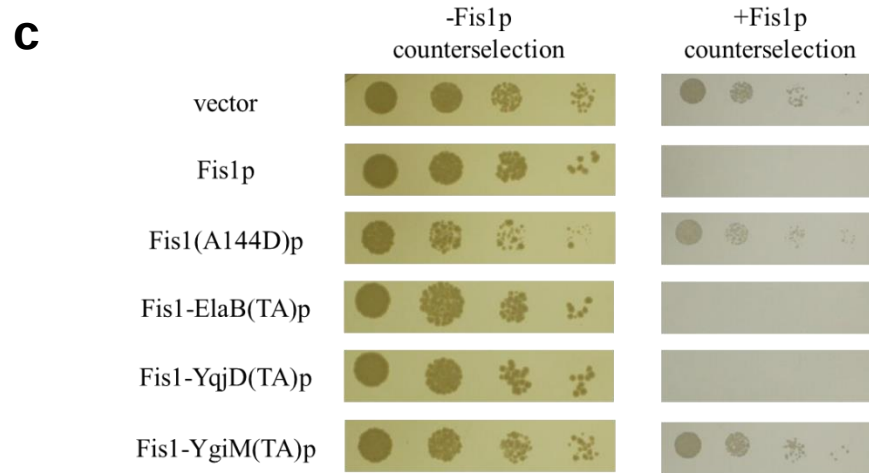


Figure 3.3 Mitochondria-localized bacterial TAs can functionally replace Fis1p TA.

a. The ElaB and YqjD TAs can replace the Fis1p TA in normal mitochondrial morphology maintenance. *fis1Δ* strain CDD741, expressing mitochondria-localized GFP from plasmid pHS12, was transformed with empty vector pRS313 or plasmids expressing wild-type Fis1p (b239), Fis1(A144D)p (b244), or Fis1p having its native TA replaced by that of ElaB (b317), YqjD (b318), or YgiM (b316) bacterial proteins. Cells were examined by fluorescence microscopy. Scale bar, 5 μ m. **b.** Quantification of mitochondrial morphology of the transformants from (a) was performed blind to genotype. White bar represents cells with fully networked mitochondria, grey bar represents cells with partially networked mitochondria, and black bar represents cells with normal mitochondrial morphology. Quantification was repeated three times ($n > 200$ per genotype), and a representative experiment is shown. **c.** Genetic assessment of Fis1p variant functionality. Strain CDD688 was transformed with the plasmids used in (a) and proliferation was assessed without selection against Fis1p activity (YPALac medium for 3 d) or following counter-selection for cells carrying functional Fis1p (SLac-His + CHX medium for 5 d).

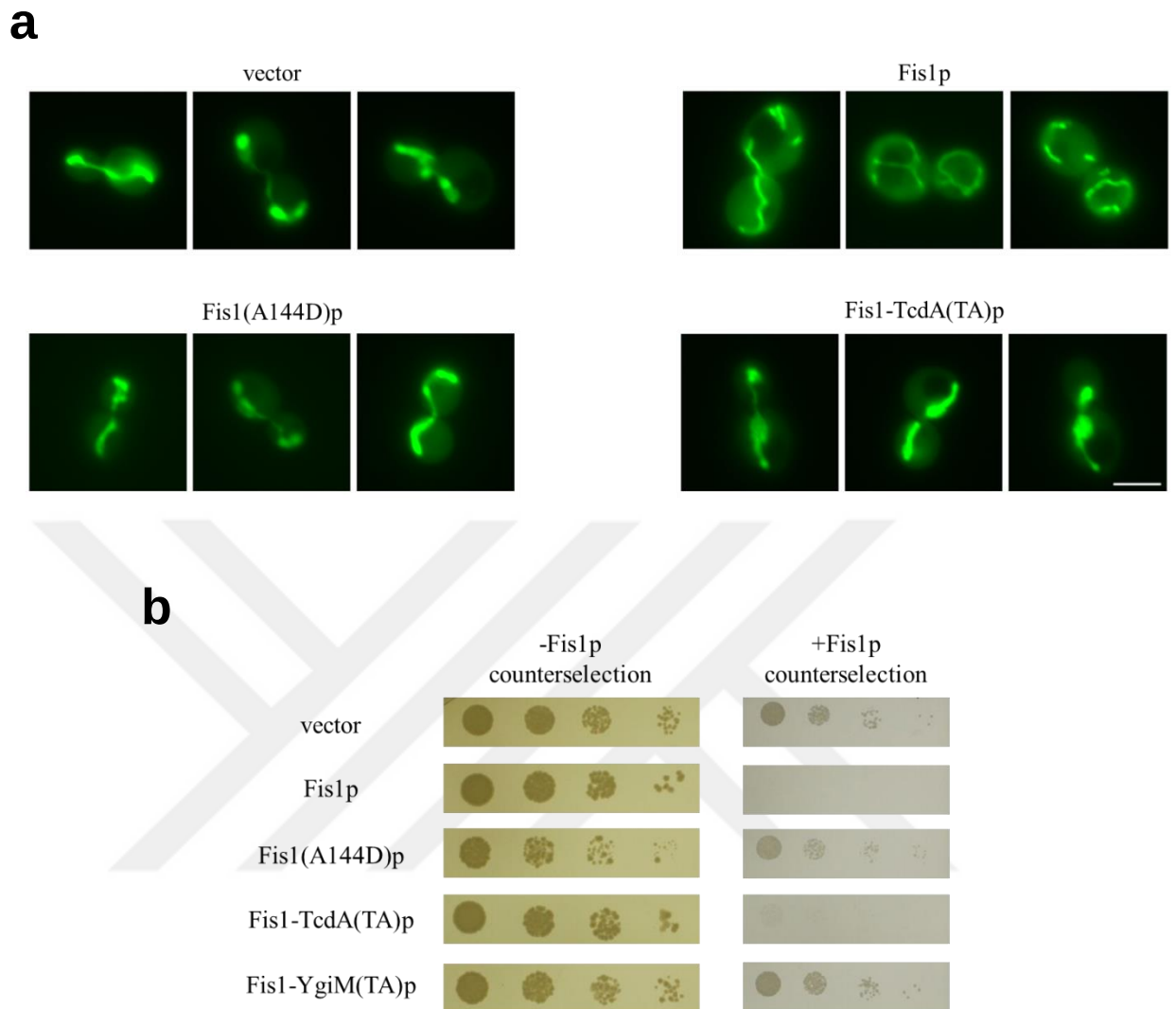


Figure 3.4 The TA of bacterial TcdA protein provides minimal localization to and function at the MOM. a. Fis1p with its own TA replaced by the predicted TcdA TA cannot provide enough Fis1p activity to restore mitochondrial morphology. *fis1Δ* strain CDD741, expressing mitochondria-localized GFP from plasmid pHS12, was transformed with empty vector pRS313 or plasmids expressing wild-type Fis1p (b239), Fis1(A144D)p (b244), or Fis1p having its native TA replaced by that of bacterial protein TcdA (b319). Cells were examined by fluorescence microscopy. Scale bar, 5 μ m. **b.** Fis1-TcdA(TA)p can allow mitochondrial division. Strain CDD688 was transformed with the plasmids used in (a) or a plasmid expressing or Fis1p having its native TA replaced by that of bacterial protein YgiM (b316) and examined as in Figure 3.3c.

3.2.3 Bacterial TAs are not recruited to the mitochondrial outer membrane via ER-mitochondria encounter sites

Endoplasmic reticulum (ER) and mitochondria are connected through an ER-mitochondria tethering complex that consists of Mdm34p, Mdm10p, Mdm12p, and Mmm1p¹⁰⁵. To rule out the possibility that bacterial TAs are distributed to mitochondrial surface through ER-mitochondrial encounter site (ERMES), we tested the localization of mCherry tagged ElaB and YqjD TAs relative to GFP tagged Mdm34p, the MOM associated component of the ER-mitochondria tethering complex. However, mCherry-ElaB(TA) and mCherry-YqjD(TA) were not restricted to ERMES labelled with Mdm34p-containing puncta (Figure 3.5). Furthermore, disruption of ERMES by deleting Mdm34p, which is essential for ERMES integrity²¹², did not alter the distribution of these fusion proteins to mitochondria that has a disturbed morphology because of Mdm34p absence (Figure 3.6a and b). These results suggest that MOM insertion of ElaB or YqjD TAs is independent of ERMES. Overall, our results represent insertion and functionality of bacterial TAs ElaB and YqjD at the MOM surface.

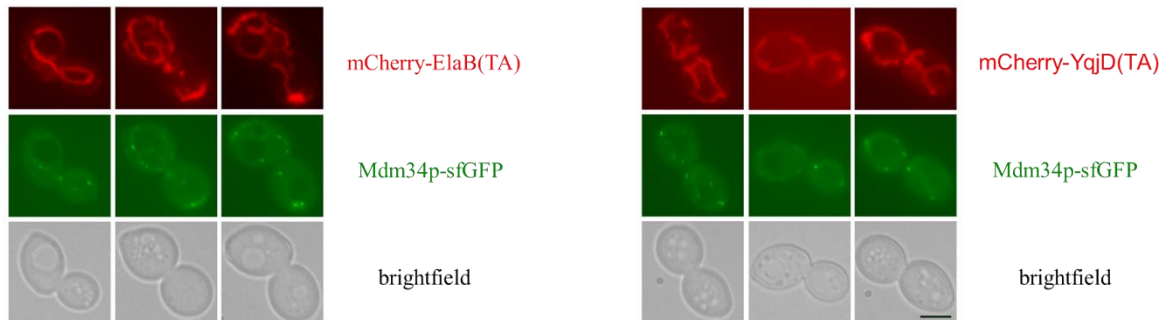
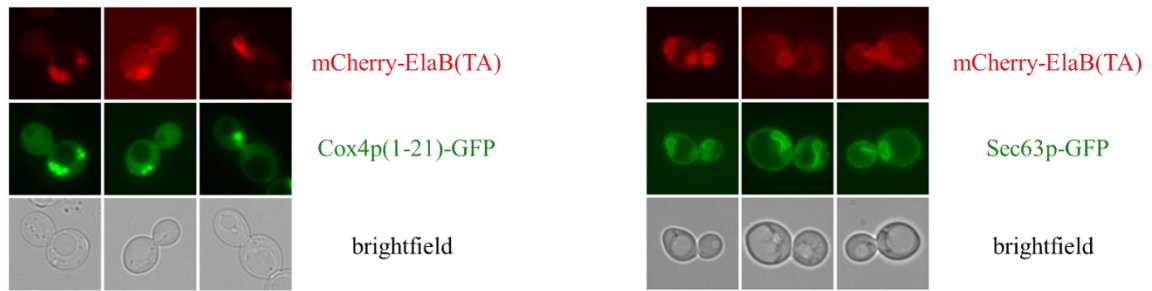


Figure 3.5 The predicted ElaB and YqjD TAs are not restricted to ERMES. Strain CDD1210, expressing Mdm34p-sfGFP, was transformed with plasmids containing mCherry-ElaB(TA) (b275) or mCherry-YqjD(TA) (b279) and visualized by fluorescence microscopy. Scale bar, 5 μ m.

a



b

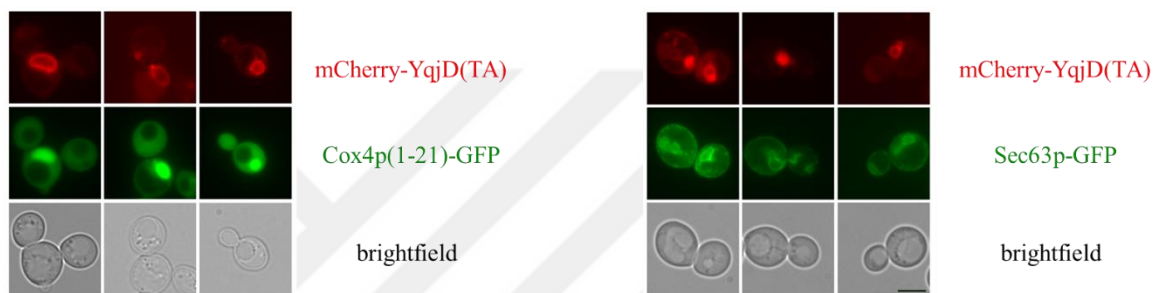


Figure 3.6 Disruption of ERMES does not affect localization of ElaB and YqjD TAs to mitochondria or ER. *mdm34Δ* strain CDD1209 was transformed with plasmids containing mCherry-ElaB(TA) (b275) (**a**) or mCherry-YqjD(TA) (b279) (**b**). The mitochondrial matrix was labelled using Cox4p(1-21)-GFP containing plasmid (pHS1), and the ER membrane was labelled using Sec63p-GFP containing plasmid pJK59. Cells were visualized by fluorescence microscopy. Scale bar, 5 μ m.

3.3 Discussion

Here we demonstrate that TAs obtained from *E. coli* can successfully insert and function at mitochondrial surface of *S. cerevisiae* cells. These findings allow us to suggest a possible scenario in which bacterial TAs assisted the insertion of an early mitochondrial translocon at the MOM at the beginning of eukaryogenesis. Fis1p is suggested to spontaneously insert at the MOM independent of any of the known mitochondrial import machineries but dependent the ergosterol content of the target membrane^{48,85}. The predicted amino acid sequences of the TAs of bacterial proteins ElaB and YqjD contain a glycine rich transmembrane domain followed by a positively charged C-terminus with di-arginine resembling that of Fis1p (Figure 3.1). Similar to Fis1p, it is likely that these bacterial TAs

could follow a spontaneous insertion mechanism although we did not represent evidence for unassisted insertion of ElaB and YqjD at the MOM. Consistent with our hypothesis, some of the members of the modern MOM translocon TOM complex are also tail-anchored ²¹³. Even though they currently do not have the ability of spontaneous insertion at the mitochondrial surface, one could suggest that these TA sequences might be remnants of an earlier self-inserting translocon derived from bacterial proteins.

There are two mechanisms that have been previously suggested for the evolution of the polytopic membrane proteins at biological membranes ⁴⁰. Accordingly, TA proteins with spontaneous insertion capability could recruit the early translocon to the lipid membrane like a chaperone and facilitate its insertion, or such proteins could cooperatively form a channel inside the lipid bilayer and initiate lateral insertion of the early translocon. Considering the intrinsic role of DUF883 protein family in *E. coli*, we favor an alternative model through co-translational import of the early translocon at the MOM ²¹⁴. YqjD functions in recruiting ribosomes to the inner membrane of *E. coli* during stationary phase, and ElaB is paralogues to YqjD. The two are expressed in response to the same cellular stress factors and bind to ribosomes in a similar manner ²¹⁵. Thus, we suggest that these proteins might have recruited ribosomes to mitochondria and upon anchoring at the MOM, they might have driven co-translational insertion of an early translocon directly into the lipid bilayer. One might wonder if thermodynamic free energy, unassisted by a translocase, can produce functionally folded membrane proteins at a lipid bilayer. Indeed, it has been demonstrated that bacterial membrane proteins can be co-translationally inserted to acquire their native folded structure at lipid bilayers that are devoid of translocases in vitro ²¹⁶. On the other hand, it was not possible to insert fully translated protein aggregates at lipid bilayers emphasizing the requirement for recruiting the ribosomes to the membrane to drive co-translational insertion, consistent with our scenario. Similar insertion of β -structured eukaryotic protein rhodopsin has also been reported ²¹⁷, suggesting that co-translational insertion of an initial mitochondrial translocon, descendants of which are currently β -barrel, at the MOM might have been possible. Although DUF883 protein family do not exist in any of the modern eukaryotic genomes, it is likely that initial translocon might have been lost, upon evolution of a more functional TOM complex. In fact, such a translocon might have been disappeared due to a likely negative selection pressure as it could interfere with the protein import through a more advanced machinery. Moreover, the presence of DUF883 family might not

even be needed as the typical sequence structure necessary for inserting proteins at the MOM might have been randomly generated through open reading frame fragments possessing a hydrophobic domain. Indeed, such peptides randomly obtained from bacteria have previously been reported to mimic mitochondrial targeting sequences²¹⁸. This view that suggests mitochondrial TAs might have been randomly generated from bacterial sequences, not intrinsically functioning in membrane targeting within bacteria, also explains why TcdA TA which does not insert at membranes in *E. coli*²¹⁹, showed partial functionality and limited localization to yeast mitochondria.

If mitochondria was evolved after the formation of the aerobic environment in the world¹³, and met the energy demand of the cell via oxidative phosphorylation¹², then there must be an unusual amount of oxidative stress for the proto-eukaryotic host, a condition that the host has not yet developed a mechanism to deal with because of its anaerobic history. In fact, this issue was also addressed in earlier theories of peroxisomal evolution, suggesting that peroxisomes were acquired through endosymbiosis to reduce the increasing level of reactive oxygen species produced upon mitochondrial evolution²²⁰, although this view later lost its popularity²²¹. Regardless of the mechanism of its evolution, it is strongly suggested that peroxisomes evolved after mitochondria²²² or even from mitochondrial membranes²⁰². If peroxisomes indeed formed after the alpha-proteobacterial uptake, then there must be a considerable amount of selective pressure for genes required for coping with the oxidative stress as a result of reactive oxygen species produced by the mitochondrial electron transport chain²²³. Interestingly, DUF883 family protein ElaB has been suggested to protect *E. coli* cells against oxidative stress upon induction by the stationary-phase sigma factor RpoS²²⁴. Belonged to the same protein family and induced by the same transcription factor, YqjD has also been suggested to protect bacteria during stress conditions, although a role for guarding the cell against oxidative environment has not been specified²¹⁵. Thus, one could speculate that upon partial relocation of alpha-proteobacterial genes to the host nucleus and before the evolution of an improved guardian against oxidative stress, ElaB and YqjD proteins might have provided a selective advantage, protecting the cell against the oxidative stress and later contributed to the formation of an early translocon at the MOM. Accordingly, absence of these proteins from the eukaryotic genome can also be explained by the decreasing selective pressure for such proteins upon formation of peroxisomes that readily detoxify reactive oxygen species in the eukaryotic genome.

As indicated by few different studies, the lipid environment of the intracellular membrane at which a TA protein will integrate at, plays critical role for its spontaneous insertion^{48,74,85}. The lipid content of the early mitochondrial membranes is especially critical for the scenario that we suggested, as in vitro studies also showed that the efficiency of unassisted co-translational insertion of membrane proteins at lipid bilayers is heavily dependent on the lipid composition²¹⁶. Thus, in order for the scenario of spontaneous insertion of the first translocon at early mitochondrial membranes to be plausible, the lipid content of the proto-eukaryote should provide a suitable milieu. Such an environment would definitely set a selective advantage, favoring nuclear encoded proteins to enter mitochondria, where they are needed, and eventually resulting in the transformation of the bacterial endosymbiont into mitochondrion⁴⁰. In vitro studies indicate that *E. coli* polar lipid extract provide a suitable environment for the efficiency of unassisted co-translational protein insertion²¹⁶. Consistently, the literature suggests that the archaeal lineages associated with the last eukaryotic common ancestor might have contained membranes resembling to those of bacteria rather than typical archaeal membranes²²⁵. Thus, this specific lipid composition of the proto-eukaryotic membranes might have been critical not only to allow proper integration of bacterial tail-anchored proteins but also to drive co-translational insertion of an early translocon at the MOM, an essential step that would let the endosymbiont fully refashion into a eukaryotic organelle, mitochondria.

Lastly, we did not investigate in detail the intracellular route of other bacterial TAs that were reported to localize to different membrane structures other than mitochondria in *S. cerevisiae*¹⁰⁶. These structures resemble ER, peroxisomal, or vacuolar membranes although confirmation with relevant markers is needed. Interestingly, a recent theory for the evolution of eukaryotic endomembrane system suggests that the first vesicles of the endomembrane system might have formed from the outer membrane vesicles secreted by the proto-mitochondrion²⁰². If that is the case, then the organelle targeting information might indeed has been obtained from the endosymbiont genome, through proteins that already have the ability to target distinct proto-mitochondrial membranes^{203,218,226}. In fact, our results in which these bacterial TAs target distinct members of the eukaryotic endomembrane system is consistent with this view. Horizontal gene transfer (HGT) is one of the powerful sources of genetic variance among prokaryotes, providing selective advantage against different environmental pressures²²⁷. Endosymbiotic gene transfer (EGT), a form of HGT, was

certainly the main factor to drive eukaryogenesis²²⁸. Accordingly, the ability to use targeting information in order to transfer proteins acquired through HGT or EGT along with other proteins evolved within the course of eukaryogenesis to different intracellular membranes where they might be best made use of, might have provided the selective advantage that would drive the evolution of the eukaryotic cell.

3.4 Conclusion

This study represents that TAs derived from bacterial proteins can target mitochondrial surface and insert at the MOM. From these results, we suggest a plausible scenario for the evolution of mitochondrial translocation machinery. Accordingly, tail-anchored proteins obtained from bacteria may have formed the initial translocon at the MOM so that proteins acquired by horizontal gene transfer could insert at different locations at the mitochondria, where they might be best utilized. Thus, presence of such translocon might have provided a selective advantage and eventually led to the evolution of the eukaryotic cell. Altogether, we hypothesize that bacterial self-inserting proteins or peptides might have generated the early mitochondrial translocon, although further experiments will be needed to test this possibility.

3.5 Materials and methods

3.5.1 Yeast strains and plasmids

Details and associated references of strains used in this study are given in Table 3.1. Construction details of plasmids and related references of the acquired plasmids are provided in Table 3.2. Oligonucleotides utilized in this work are listed in Table 3.3.

3.5.2 Media and culture conditions

Synthetic complete (SC) medium consists of 0.67% yeast nitrogen base lacking amino acids, 0.1% casamino acids, 2% dextrose, 50 µg/ml adenine hemisulfate, and either 100

µg/ml L-tryptophan (SC-Ura) or 25 µg/ml uracil (SC-Trp). Supplemental minimal medium (SMM) consists of 0.67% yeast nitrogen base lacking amino acids, 2% dextrose, 20 µg/ml adenine hemisulfate, 20 µg/ml methionine, 20 µg/ml uracil, 30 µg/ml lysine. SMM also contains 20 µg/ml histidine, 100 µg/ml leucine, and/or 20 µg/ml tryptophan depending on selection condition. YPALac medium contains 1% yeast extract 2% peptone, 1.2% NaOH, and a volume of lactic acid to adjust the pH to 5.5. SLac medium devoid of histidine consist of 0.67% yeast nitrogen base lacking amino acids, 1.2% NaOH, a volume of lactic acid to adjust the pH to 5.5, 20 µg/ml uracil, 20 µg/ml adenine hemisulfate, 20 µg/ml methionine, 20 µg/ml tryptophan, 30 µg/ml lysine, and 100 µg/ml leucine. Solid media also contain 1.7% bacteriological agar. Cells were incubated at 30°C unless indicated otherwise. For serial dilution assays, strains in logarithmic proliferation phase were diluted to an OD₆₀₀ of 0.1, and 4 µl of this dilution and three serial five-fold dilutions were spotted to a solid medium.

3.5.3 Microscopy

For epifluorescence microscopy studies, cells grown to logarithmic phase of proliferation were examined by using an Eclipse 80i microscope with a 100X Plan Fluor objective and linked to a DS-Qi1Mc camera (Nikon, Tokyo, Japan). Cells were grown in SMM medium suitable for plasmid selection. Images were captured using NIS-Elements version of AR 3.2, and exposure times were set automatically. mCherry fusions are driven by the ADH1 promoter and universally contain Fis1p amino acids 119-128 (not necessary or sufficient for mitochondrial targeting) linking mCherry to each TA. The brightness of all images of mCherry expression was adjusted in Adobe Photoshop CS5 (Adobe, San Jose, California) to an equivalent extent. Microscopic assessment of Fis1p variant functionality was performed by scoring mitochondrial morphology blind to genotype.

3.5.4 Genetic assessment of Fis1p insertion and functionality

Cells unable to perform mitochondrial fusion lose mitochondrial DNA (mtDNA) and cannot survive on non-fermentable medium because of the ongoing division events, unless mitochondrial division is also blocked⁹⁸. In strain CDD688²²⁹ *FZO1* and *FIS1* genes are

deleted and the strain contains a CHX-counter-selectable plasmid expressing Fzo1p. Upon counter-selection for the plasmid expressing Fzo1p, cells will not lose mtDNA and ability to proliferate on non-fermentable medium unless functional Fis1p is expressed to drive mitochondrial division. To test the functionality of each Fis1p variant, in which its native is replaced with different bacterial TAs, strain CDD688 was transformed with plasmids expressing wild-type Fis1p or variants containing different bacterial TAs. Transformants were cultured for 3 days in YPALac medium lacking CHX to permit cells to lose the FZO1-encoding plasmid. Serial dilutions were then spotted to SLac –His + 3 µg/mL CHX (“lactate/no fusion”) and incubated for 5 days to test for maintenance of mtDNA following counter-selection for FZO1, with cell proliferation indicating a lack of Fis1p function.

Table 3.1 Strains used in Chapter 3

Strain	Genotype	Source	Parental Strain	Method Used
BY4743	<i>MATa/α his3Δ1/ his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0</i>	EUROSCARF Y20000		
MaV203	<i>MATa leu2-3,112; trp1-901; his3Δ200; ade2-101 (?); cyh2; can1; gal4Δ; gal80Δ; GAL1::lacZ; HIS3_{UASGAL1}::HIS3@LYS2; SPAL10_{UASGAL1}::URA3</i>	Invitrogen		
CDD463	<i>MATa leu2Δ0 his3Δ200 met15Δ0 trp1Δ63 ura3Δ0</i>	229		
CDD688	<i>MATa ura3-1 trp1Δ2 ade2-1 leu2-3,112 his3-11,15 can1-100 cyh2</i>	230		

	<i>fzo1Δ::LEU2</i> <i>fis1Δ::kanMX4</i> + plasmid b19 [pFZO1-CYH2-TRP1]			
CDD741	<i>MATa ura3-1 trp1Δ2 ade2-1 leu2-3,112 his3-11,15 can1-100 fis1Δ::kanMX4</i> + plasmid pHS12 [pCOX4(1-21)-GFP-LEU2]	94		
BMA64	<i>MATa/α ura3-1/ura3-1 trp1Δ2/trp1Δ2 ade2-1/ade2-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 can1-100 /can1-100</i>	EUROSCARF 20000D		
CDD1208	<i>MATa/α ura3-1/ura3-1 trp1Δ2/trp1Δ2 ade2-1/ade2-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 can1-100 /can1-100 mdm34Δ::hphNT1/MDM34</i>	constructed by Dr. C. Dunn	BMA64	<i>MDM34</i> was deleted using primers 1093/1094 and template b80.
CDD1209	<i>MATa ura3-1 trp1Δ2 ade2-1 leu2-3,112 his3-11,15 can1-100 mdm34Δ::hphNT1</i>	constructed by Dr. C. Dunn	CDD1208	Sporulation
CDD1210	<i>MATa/α his3Δ1/ his3Δ1 leu2Δ0/leu2Δ0 met15Δ0/MET15 LYS2/lys2Δ0 ura3Δ0/ura3Δ0 MDM34-sfGFP::SpHIS5/MDM34</i>	constructed by Dr. C. Dunn	BY4743	<i>MDM34</i> was tagged with superfolder GFP using primers 1388/1389 and template b93.

Table 3.2 Plasmids used in Chapter 3

Plasmid	Name	Source	Construction details or source
pRS313	pARS-CEN-HIS3	231	
pRS314	pARS-CEN-TRP1p	231	
pRS315	pARS-CEN-LEU2	231	
pRS316	pARS-CEN-URA3p	231	
pHS1	pCOX4(1-21)-GFP-HIS3	231	
pHS12	pCOX4(1-21)-GFP-LEU2	231	

pJK59	pSEC63- <i>GFP-URA3p</i>	232	
pKS1	pTRP1- <i>CYH2</i>	233	
b80	pRS306H	234	
b93	pFA6a-link- yoSuperfolderGFP-SpHis5	235	
b100	pCYH2- <i>TRP1-GAL4- sfGFP-FIS1</i>	94	
b101	pCYH2- <i>TRP1-GAL4- sfGFP-FIS1(ΔTA)</i>	94	
b109	pLEU2- <i>mCherry-FIS1(TA)</i>	94	

b141	pURA3-GAL4-sfGFP-FIS1	constructed by A. Keskin	The insert of plasmid b100 was amplified using primers 698 and 517. The PCR product was co-transformed with <i>NotI</i> -cut pRS316 into strain BY4743.
b152	pTRP1-mCherry-FIS1(TA)	constructed by A. Keskin	The insert of plasmid b109 was amplified using primers 11 and 12. The PCR product was co-transformed with <i>NotI</i> -cut pRS314 into strain CDD463.
b180	pCYH2-TRP1-GAL4-sfGFP-FIS1 (A144D)	94	
b238	pCYH2-TRP1-FIS1	94	
b239	pHIS3-FIS1	94	
b244	pHIS3-FIS1 (A144D)	94	
b274	pLEU2-mCherry-ygiM(TA)	constructed by A. Keskin	The <i>ADH1</i> promoter and mCherry was amplified from plasmid b152 using primers 11 and 764 The region encoding the predicted tail anchor of YgiM was amplified from <i>Escherichia coli</i> strain DH5α using primers 963 and 964. The <i>ADH1</i> 3' UTR was amplified from plasmid b152 using primers 775 and

			12. PCR products were co-transformed with <i>NotI</i> -cut pRS315 into strain BY4743.
b275	pLEU2-mCherry-elaB(TA)	constructed by A. Keskin	The <i>ADH1</i> promoter and mCherry was amplified from plasmid b152 using primers 11 and 764 The region encoding the predicted tail anchor of ElaB was amplified from <i>Escherichia coli</i> strain DH5 α using primers 953 and 954. The <i>ADH1</i> 3' UTR was amplified from plasmid b152 using primers 775 and 12. PCR products were co-transformed with <i>NotI</i> -cut pRS315 into strain BY4743.
b279	pLEU2-mCherry-yqjD(TA)	constructed by A. Keskin	The <i>ADH1</i> promoter and mCherry was amplified from plasmid b152 using primers 11 and 764 The region encoding the predicted tail anchor of YqjD was amplified from <i>Escherichia coli</i> strain DH5 α using primers 967 and 968. The <i>ADH1</i> 3' UTR was amplified from plasmid b152 using primers 775 and 12. PCR products were co-transformed with <i>NotI</i> -cut pRS315 into strain BY4743.
b281	pLEU2-mCherry-tcdA(TA)	constructed by A. Keskin	The <i>ADH1</i> promoter and mCherry was amplified from plasmid b152 using primers 11 and 764 The region encoding the predicted tail anchor of TcdA was amplified from <i>Escherichia coli</i> strain DH5 α using primers 957 and 958. The <i>ADH1</i> 3' UTR was amplified from plasmid b152 using primers 775 and 12. PCR products were co-transformed with <i>NotI</i> -cut pRS315 into strain BY4743.

b313	pCYH2-TRP1-GAL4-sfGFP-FIS1 (<i>elaB</i> -TA)	This study	A product containing the <i>FIS1</i> promotor, the <i>GAL4</i> ORF, the superfolderGFP ORF, and a portion of <i>FIS1</i> was amplified from plasmid b141 using primers 11 and 984. A second product encoding the ElaB tail anchor was amplified from plasmid b275 using primers 953 and 954. The <i>ADH1</i> 3' UTR was amplified from plasmid b141 using primers 775 and 12. PCR products were co-transformed with <i>NotI</i> -cut pKS1 into competent <i>Saccharomyces cerevisiae</i> cells.
b314	pCYH2-TRP1-GAL4-sfGFP-FIS1 (<i>yqjD</i> -TA)	This study	A product containing the <i>FIS1</i> promotor, the <i>GAL4</i> ORF, the superfolderGFP ORF, and a portion of <i>FIS1</i> was amplified from plasmid b141 using primers 11 and 984. A second product encoding the YqjD tail anchor was amplified from plasmid b279 using primers 967 and 968. The <i>ADH1</i> 3' UTR was amplified from plasmid b141 using primers 775 and 12. PCR products were co-transformed with <i>NotI</i> -cut pKS1 into competent <i>Saccharomyces cerevisiae</i> cells.
b316	pHIS3-FIS1- <i>ygiM</i> (TA)	This study	The <i>FIS1</i> promoter and open reading frame lacking the Fis1p tail anchor was amplified from plasmid b238 using primers 11 and 984. The region encoding the predicted tail anchor of YgiM was amplified from plasmid b274 using primers 963 and 964. The <i>ADH1</i> 3' UTR was amplified from plasmid b238 using primers 775 and 12. PCR products were co-transformed with <i>NotI</i> -cut pRS313 into competent <i>Saccharomyces cerevisiae</i> cells.
b317	pHIS3-FIS1- <i>elaB</i> (TA)	This study	The <i>FIS1</i> promoter and open reading frame lacking the Fis1p tail anchor was amplified from plasmid b238 using primers 11 and 984. The region encoding the predicted tail anchor of ElaB was amplified from

			plasmid b275 using primers 953 and 954. The <i>ADH1</i> 3' UTR was amplified from plasmid b238 using primers 775 and 12. PCR products were co-transformed with <i>NotI</i>-cut pRS313 into competent <i>Saccharomyces cerevisiae</i> cells.
b318	<i>pHIS3-FIS1-yqjD(TA)</i>	This study	The <i>FIS1</i> promoter and open reading frame lacking the Fis1p tail anchor was amplified from plasmid b238 using primers 11 and 984. The region encoding the predicted tail anchor of YqjD was amplified from plasmid b279 using primers 967 and 968. The <i>ADH1</i> 3' UTR was amplified from plasmid b238 using primers 775 and 12. PCR products were co-transformed with <i>NotI</i>-cut pRS313 into competent <i>Saccharomyces cerevisiae</i> cells.
b319	<i>pHIS3-FIS1-tcdA(TA)</i>	This study	The <i>FIS1</i> promoter and open reading frame lacking the Fis1p tail anchor was amplified from plasmid b238 using primers 11 and 984. The region encoding the predicted tail anchor of TcdA was amplified from plasmid b281 using primers 957 and 958. The <i>ADH1</i> 3' UTR was amplified from plasmid b238 using primers 775 and 12. PCR products were co-transformed with <i>NotI</i>-cut pRS313 into competent <i>Saccharomyces cerevisiae</i> cells.

Table 3.3 Oligonucleotides used in Chapter 3

Name	Sequence (5'-3')
11	GTAAAACGACGGCCAGTGAATTG
12	CGCCAAGCTCGGAATTAACCCTCAC
517	ATATCGAATTCCTGCAGCCCGGGGGATCCATGCCGGTAGAGGTGTGGTCAATAAGAGCG A

698	GACTCACTATAGGGCGAATTGGAGCTCCACCAGTTCAAATAACATGTGTCCATTACCTGT
764	TGTTTCCTTCTGGATCTTATCCTCTACCATCTTGTACAGCTCGTCCATGCCGCCGGTGGA
775	TAAACACTTCTAAATAAGCGAATTTCTTATGATTTATGAT
953	ATGGTAGAGGATAAGATCCAGAAGGAAACACCCTGGCAAGGAATTGGTGTGGGCGCGGC C
954	ATCATAAGAAATTCGCTTATTTAGAAGTGTTTAACGGCGTGCCAGCAACAGTCCTAGTAC
957	ATGGTAGAGGATAAGATCCAGAAGGAAACAGCATCTGGATTGCGCGGCAACGATGGT G
958	ATCATAAGAAATTCGCTTATTTAGAAGTGTTTAACCCTGACGCGCCGCTTTGCGCATCAT
963	ATGGTAGAGGATAAGATCCAGAAGGAAACATGGTTTATGTATGGCGGTGGCGTGCTGGG G
964	ATCATAAGAAATTCGCTTATTTAGAAGTGTTTAGTTCATCCAGCGATCTTTGCGTTTGCG
967	ATGGTAGAGGATAAGATCCAGAAGGAAACATGGACGGGCGTGGGCATTGGCGCTGCAAT C
968	ATCATAAGAAATTCGCTTATTTAGAAGTGTTTAACGACGCGACAGCAGAACGCCGAGCA C
984	TGTTTCCTTCTGGATCTTATCCTCTACCAT
1093	TAACAAAAAAAAACACAACCTGCAAGAACTTCAGCATCGTCAGATTGTACTGAGAGTGCA C
1094	ATGTATTTGTGTAGTTATGTACTTAGATATGTAACCTAATCTGTGCGGTATTCACACCG
1388	CTGGAAATGGGGCATGGAGGATAGCCCCCACCATATCATGGTGACGGTGCTGGTTTA
1389	ATGTATTTGTGTAGTTATGTACTTAGATATGTAACCTAATTCGATGAATTCGAGCTCG

Chapter 4

BACTERIAL TAIL ANCHOR YGIM AS A TOOL TO TRACK PEROXISOMAL TRAFFICKING

4.1 Introduction

Peroxisomes are organelles taking role in various cellular functions including metabolism of fatty acids ²³⁶ and detoxification of hydrogen-peroxide to protect cells from oxidative damage ²³⁷. The enzyme content of peroxisomal matrix enables adaptation of the organism to unique environments, by metabolizing distinct substrates ¹¹¹. Also, number of yeast peroxisomes could change depending on the carbon source of the growth media. Growth on oleic acid for most of the yeast strains and on methanol for methylotrophic yeasts as the sole carbon source depends on peroxisomes ²³⁸. The severity of symptoms caused by peroxisomal disorders, such as Zellweger-Syndrome, highlights the importance of functional peroxisomes in humans as well. Mutations in genes coding for peroxisomal proteins called peroxins is the underlying genetic defect, causing patients to suffer on neurologic, hepatic, and renal dysfunctions ^{112,239}.

Unlike rest of the single-membraned organelles, peroxisomes are capable of importing proteins directly from the cytosol ¹¹³. These proteins include matrix proteins, import of which is facilitated by peroxisomal membrane proteins (PMPs) and PMPs imported through a process that is less understood compared to matrix protein import ¹²⁰. PMPs constitute the protein import machinery that facilitates the peroxisomal protein import cycle. This cycle includes recognition of the peroxisomal targeting signals of type 1 and 2 (PTS1 and PTS2) by the cytosolic receptors, interaction of the receptor-cargo complex with the docking

complex proteins found on the peroxisomal membrane, release of the cargo to the matrix space through a translocation pore, and recycling of the receptor back to the cytosol ¹¹⁰.

Among 24 different peroxins that take role in peroxisomal protein import, Pex3p and Pex19p in the yeast *Saccharomyces cerevisiae* (also PEX16 in mammals) are involved in the import of other PMPs. Defects in rest of the PMPs cause mislocalization of matrix proteins to the cytosol and PMPs are found on peroxisomal membranes called membrane ghosts. Because of the specific role of Pex3p and Pex19p in PMP import, earlier assumption was that *pex3Δ* or *pex19Δ* yeast mutants are devoid of peroxisomal ghosts ^{120,162}. However, there is evidence of peroxisomal membranes capable of accepting several different PMPs in *pex3Δ* or *pex19Δ* yeast mutants ^{116,117}. Furthermore, reintroduction of either of the two proteins into in *pex3Δ* or *pex19Δ* cells, allows formation of mature peroxisomes suggesting that peroxisomes can form *de novo* ¹⁷². Thus, although Pex3p and Pex19p are involved in direct import of PMPs, there is at least one other alternative route for PMP insertion, via the endoplasmic reticulum (ER).

Pex15p, yeast homolog of mammalian PEX26, is the only known tail-anchored PMP functioning in peroxisomal protein import in the yeast *S. cerevisiae* ^{93,191}. With several other PMPs, it was represented to traffic to peroxisomes via the ER ¹¹¹. In fact, GET pathway, the main pathway that facilitates the insertion of TA proteins at ER membranes ⁷¹, is suggested to have a role in PEX15p targeting to peroxisomes ⁶². On the other hand, there is evidence of alternative pathways for TA protein targeting at the ER, such as recently discovered SND pathway ⁶⁸. Interestingly, deletion of GET pathway components in *S. cerevisiae*, results in mislocalization of the Pex15p at the MOM, although a possible insertion mechanism has not been suggested ⁷¹.

Inserting directly from the cytosol or through ER derived vesicles of varying maturity stages, peroxisomal proteins seem to have many alternative pathways to take and peculiar peroxisomal membranes to insert at. Yet, this entire peroxisomal trafficking is not completely understood. Although there are fluorescently tagged peroxisomal markers to follow this traffic, they are either matrix-localized and can only target import competent mature peroxisomes, or disrupt peroxisomal biogenesis as they are native membrane proteins ¹⁶⁹. In order to overcome this obstacle, we hypothesized that TA of bacterial protein YgiM, which was previously reported to localize to mature peroxisomes ¹⁰⁶, might be a useful tool to track TA protein targeting at peroxisomal membranes. Indeed, when expressed in yeast *S.*

cerevisiae, TA of YgiM protein functionally replaced Pex15p TA and inserted at different peroxisomal membranes. Thus, we suggest that YgiM TA with no intrinsic peroxisomal function that would interfere with organelle biogenesis could be used to follow peroxisomal protein trafficking in yeast.

4.2 Results

4.2.1 TA of bacterial protein YgiM can insert and function at peroxisomal membranes

YgiM TA was reported to have a intracellular puncta distribution similar to that of peroxisomes ¹⁰⁶ and later verified to co-localize with peroxisome targeted superfolder GFP (sfGFP) (C. Dunn, unpublished results) when fused to the C-terminus of fluorescent protein mCherry. Thus, we hypothesized that TA of *Escherichia coli* protein YgiM can be used to track peroxisomal protein trafficking in *S. cerevisiae* cells. The predicted TA domain of YgiM compared to *S. cerevisiae* Fis1p, Pex15p, and human PEX26 TAs is shown in Figure 4.1. Additionally, we also included the TA of ER-localized isoform of human cytochrome b5 for comparison, as this TA domain has been reported to mediate unassisted insertion of cytochrome b5 at the ER membrane ⁴⁹ (Figure 4.1). To assess the probability of each of these proteins to form transmembrane helices, we also analyzed their structural and sequence characteristics. As seen in Figure 4.2, the probability of YgiM to form a transmembrane domain is quite higher compared to other peroxisome-specific TA proteins Pex15p and PEX26, while being similar to that of ER-localized cytochrome b5 and Fis1p that dual localizes to mitochondria and peroxisomes. On the other hand, the C-terminal TA region of YgiM do not resemble any of these proteins.

To test the proper integration of YgiM TA at peroxisomal membranes, we replaced the native TA of Pex15p PMP, a tail-anchored PMP found in *S. cerevisiae* ⁸⁴, with YgiM TA and expressed this protein in *pex15Δ S. cerevisiae*. Earlier studies have reported that deletion of Pex15p in *S. cerevisiae* provokes loss of import competent peroxisomes, and matrix targeted enzymes mislocalize to the cytosol, although these mutants still harbor peroxisomal membrane ghosts that possess PMPs ⁸⁴. As expected, expression of Pex15(1-331)p lacking

the entire TA domain in *pex15Δ* mutants did not restore the wild-type peroxisomal morphology, suggested by the cytosolic distribution of matrix-localized sfGFP, while the C-terminus of Pex15(1-331)p fused to its native TA restored wild-type peroxisomal distribution in most of the cells (Figure 4.3a and b). Interestingly, replacing the native TA of Pex15p with that of bacterial protein YgiM, restored the wild-type peroxisomal distribution, although not as strong as native Pex15p TA. A control TA obtained from *E. coli* YqjD protein that targets the MOM (see Chapter 3) could not restore Pex15p activity, a result that is consistent with functional integration of bacterial YgiM TA at peroxisomal membranes, similar to a native PMP TA.

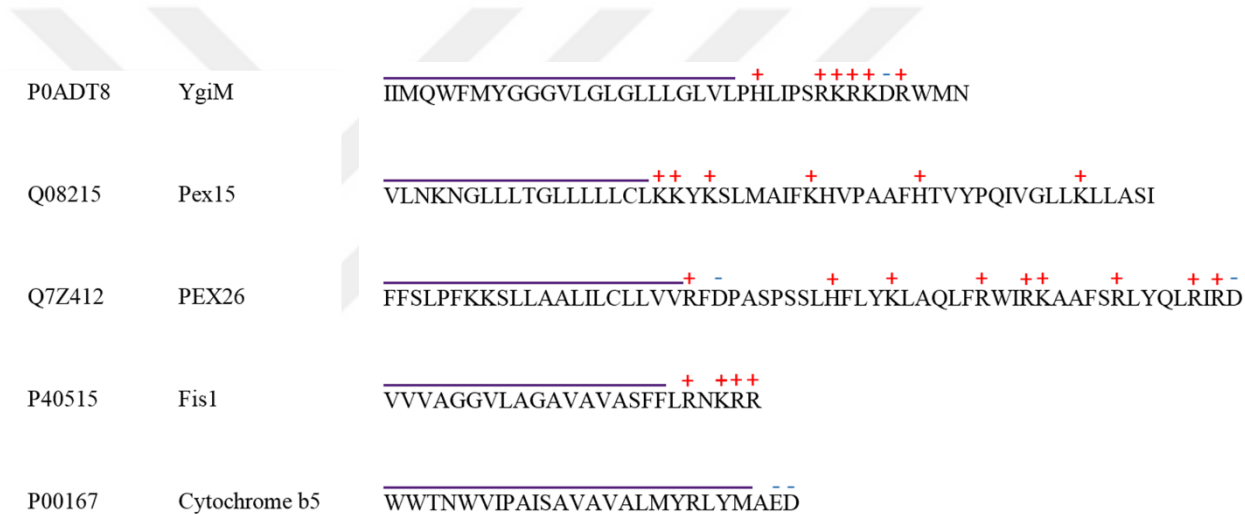
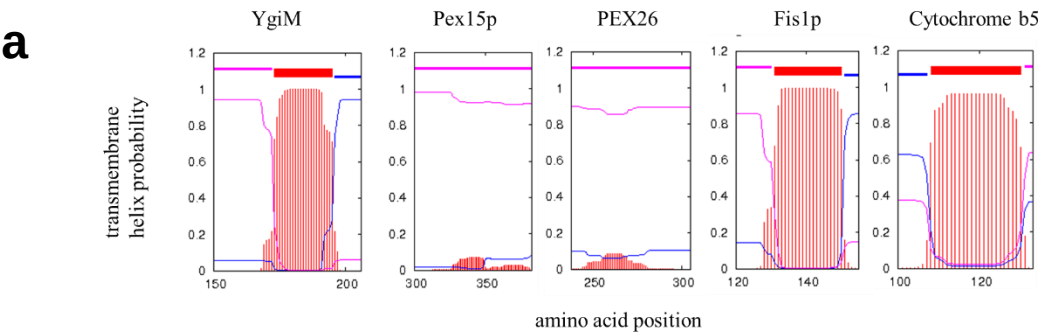


Figure 4.1 Predicted TA of YgiM. The UniProt accession numbers and names of selected proteins that the TAs are belonged to are provided, along with the sequences of the predicted TAs. The single predicted membrane-passing domain is shown by a purple line. Charged amino acids are also indicated. The relevant portion of *S. cerevisiae* Fis1p, Pex15p, PEX26, and human cytochrome b5 are also shown for sequence comparison.

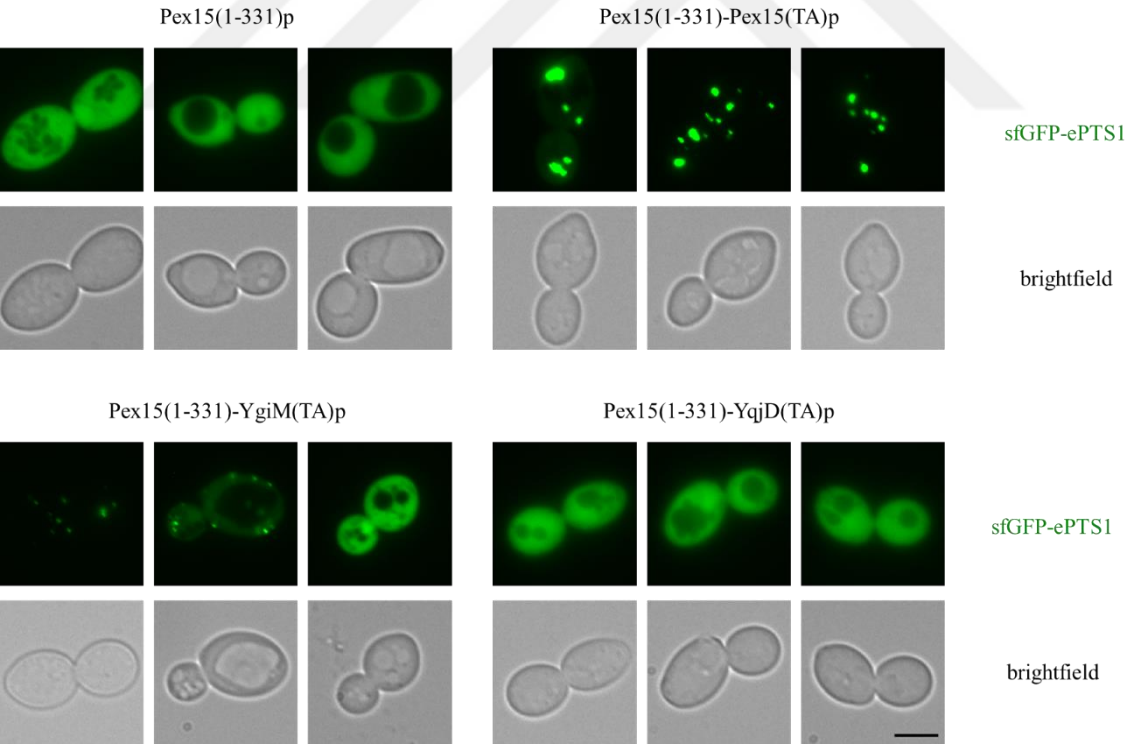


b

P0ADT8	YgiM	IIMQWFMYGGGVIGLGLLLGLVLP	PHLIPSRKRKDRW	----	MN	-----
Q08215	Pex15p	-----	VLNKNGLLLTGLLLLCLK	-----	KYKSL	-----MAIFKHV-PAAFHTVYPQIVGLLKLLASI
Q7Z412	PEX26	---	FFSLPFKKSLLAALILCLLVV	RFDPASPSLHFLYKLAQLFRWIRKAAFSRLYQLRIRD	-----	
P40515	Fis1p	-----	VVAGGVLAGAVAV	----	ASFF	LRNKRR-----
P00167	cytochrome b5	WWTNWVIPAISAVAVALMYRLYM	AE	----	D	-----

Figure 4.2 Structural and sequence characteristics of the YgiM TA. **a.** The YgiM TA is predicted to be a transmembrane helix. The indicated C- terminal amino acid positions of each protein was tested using the TMHMM Server version 2.0 ²⁴⁰, and individual amino acid scores were plotted. The TA proteins that localizes to peroxisomes, (Pex15p, PEX26, and Fis1p), mitochondria (Fis1p), or ER (cytochrome b5) were also tested for comparison. The cytosolic, transmembrane, and organellar portion of each protein is shown with pink, red, and blue, respectively. **b.** YgiM tail anchor do not resemble other tail-anchored proteins listed in (a). The predicted transmembrane domain and the following C-terminal portion of tail-anchored proteins were aligned using Clustal Omega (Soding 2005). The transmembrane domain is colored red.

a



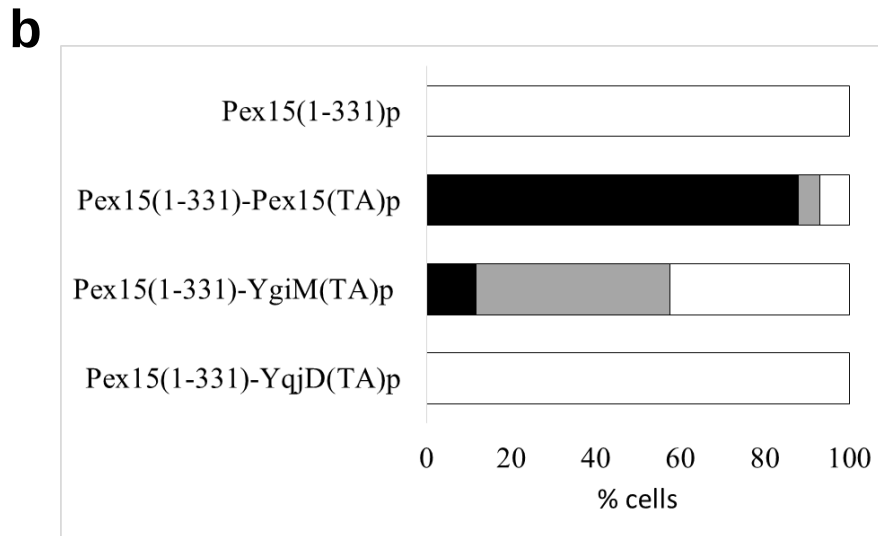


Figure 4.3 Peroxisome-localized bacterial YgiM TA can replace Pex15p TA functionality. **a.** The YgiM TA can replace the Pex15p TA in promotion of import competent peroxisomes. *pex15Δ* strain CDD1182, expressing peroxisomal matrix-localized sfGFP, was transformed with plasmids expressing Pex15(1-331)p lacking Pex15p TA (b341), Pex15(1-331)p fused to Pex15p TA (b326), or to bacterial YgiM (b329) or YqjD (b330) TAs. Cells were examined by fluorescence microscopy. Scale bar, 5 μ m. **b.** Quantification of peroxisomal morphology of the transformants from (a) was performed blind to genotype. White bar represents cells lacking import competent peroxisomes, grey bar represents cells with import competent peroxisomes, but competent to a less extent than wild-type cells, and black bar represents cells with import competent peroxisomes. Quantification was repeated three times ($n > 200$ per genotype), and a representative experiment is shown.

4.2.2 YgiM TA labels ER associated peroxisomal membranes

Bacterial YgiM TA, containing an mCherry fusion at its N-terminus was observed to successfully label early peroxisomal vesicles in *pex3Δ* or *pex19Δ* mutants, confirmed with its co-localization with GFP labelled Pex14p puncta (C. Dunn, unpublished results), a PMP protein that is suggested to localize to pre-peroxisomal membranes in *pex3Δ* or *pex19Δ* mutants^{116,117}. To confirm that these Pex14p puncta are indeed intermediate vesicles between ER and mature peroxisomes, we decided to observe their intracellular localization compared to the ER membranes. For this purpose, we expressed Sec63p-mRFP fusion

protein to label ER in a yeast strain that expresses Pex14p containing sfGFP on its C-terminus. Indeed, sfGFP labelled Pex14p puncta seems to be near the ER membranes in wild-type cells, although this observation should be confirmed with high-resolution microscopy techniques (Figure 4.4). Furthermore, these Pex14p puncta were also visible in *pex3Δ* or in *pex19Δ* yeast strains with the same genetic background, suggesting that mCherry-YgiM(TA) fusion protein could label early peroxisomal membranes that are near the ER in *pex3Δ* or *pex19Δ* mutants.

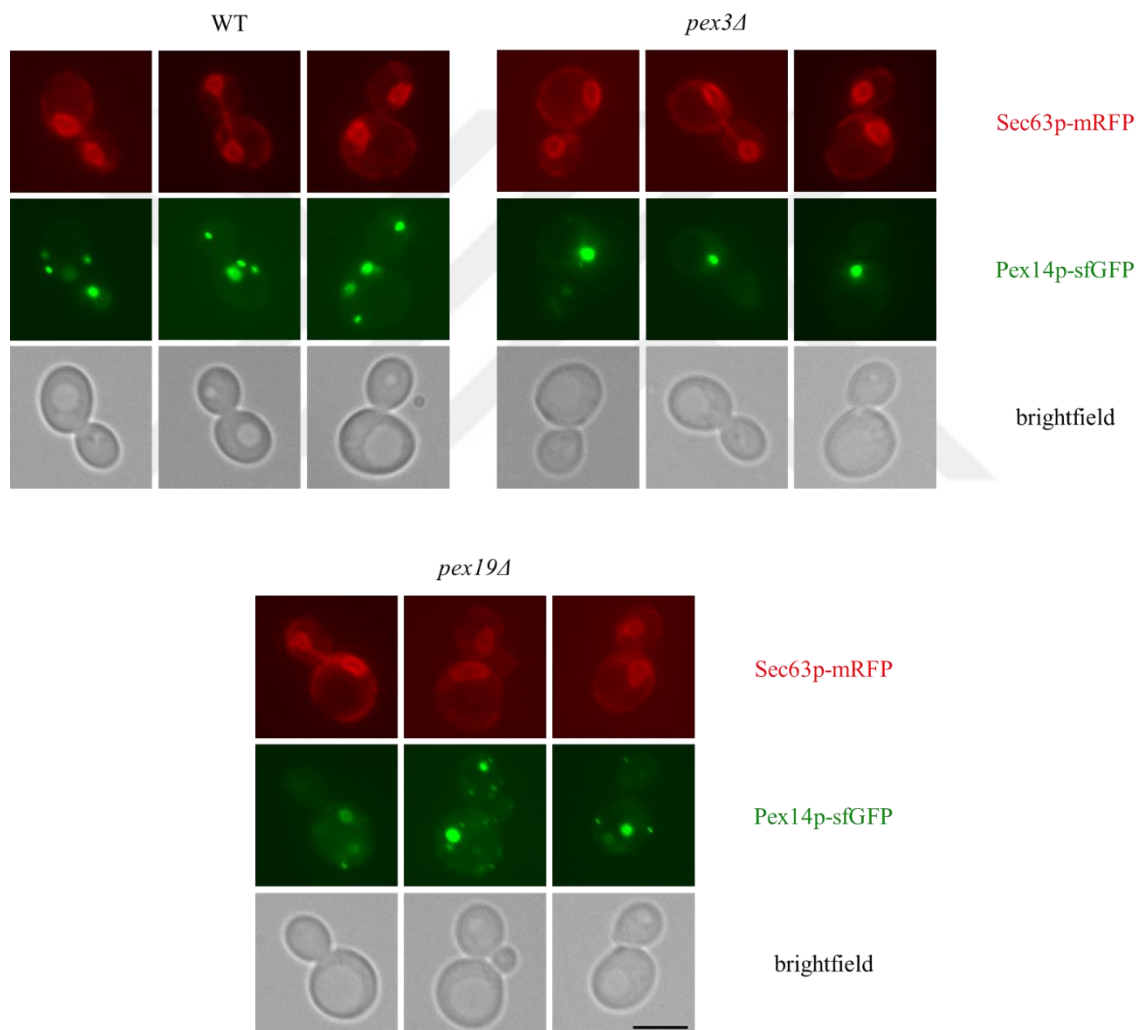


Figure 4.4 Peroxisome-localized Pex14p-sfGFP is in near ER membranes. Pex14p-sfGFP fusion protein labels immature peroxisomal vesicles that are near the ER. WT, *pex3Δ*, or *pex19Δ* strains CDD1200, CDD1201, or CDD1202 expressing Pex14p-sfGFP, were transformed with a plasmid expressing ER-localized mRFP (b212). Cells were examined by fluorescence microscopy. Scale bar, 5 μ m.

4.2.3 Pex15p localization to peroxisomes is not strictly dependent on Spf1p

Previous studies propose that deletion of an ER ATPase of unknown function Spf1p, results in mislocalization of the MOM TA protein Fis1p to ER by converting the ergosterol content of ER membrane similar to that of MOM ⁴⁸. Similarly, expression of mCherry-YgiM(TA) in *spf1Δ S. cerevisiae*, provoked a dual localization both at ER like membranes and ER-associated peroxisomal puncta, although ER localization is yet to be confirmed with an ER marker (C. Dunn, unpublished results). According to these results, one could suggest that changing the lipid composition of ER membrane causes mislocalization of mCherry-YgiM(TA) to ER and ER-associated peroxisomal membranes. To assess whether such a phenotype could also be observed with full length Pex15p containing mCherry on its N-terminus, we expressed mCherry-Pex15p fusion protein along with peroxisomal matrix targeted sfGFP-ePTS1 in a *spf1Δ* yeast strain. Unlike mCherry-YgiM(TA), mCherry-Pex15p fusion protein did not localize to ER-like membranes, instead it was distributed to sfGFP-ePTS1 labeled peroxisomes (Figure 4.5). Notably, some portion of the cells had dramatically altered peroxisomal phenotype confirmed with the cytosolic distribution of sfGFP-ePTS1. In cells with such altered peroxisomal phenotype, mCherry-Pex15p distribution seems to be mitochondrial in wild-type cells, whereas this distribution shifts towards ER-like membranes in *spf1Δ* cells. mCherry-Pex15p seems to localize to mitochondrial membranes in wild-type cells and ER membranes in *spf1Δ* mutants only in the absence of mature peroxisomes while mCherry-Pex15p prefers peroxisomal membranes when mature peroxisomes are present. On the other hand, mCherry-YgiM(TA) is more equally distributed at both ER and peroxisomal membranes in *spf1Δ* cells (C. Dunn, unpublished results), similar to Fis1p ⁴⁸. Also, we note that Pex15p is not under its native promoter and overexpression of a PMP fused to a fluorescent tag disturbed peroxisomal biogenesis, consistent with the previous literature ¹⁶⁹, although we did not quantify the extent of this disruption.

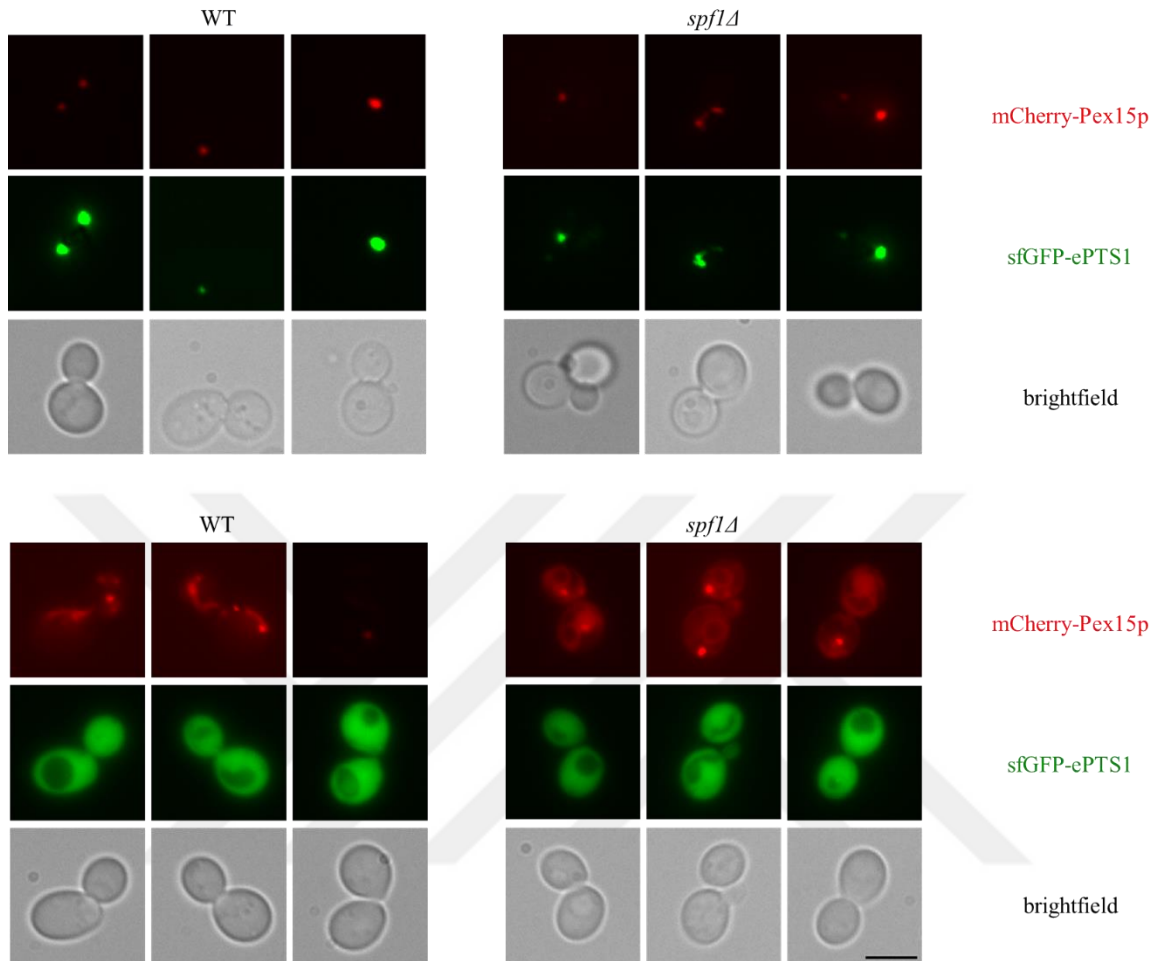
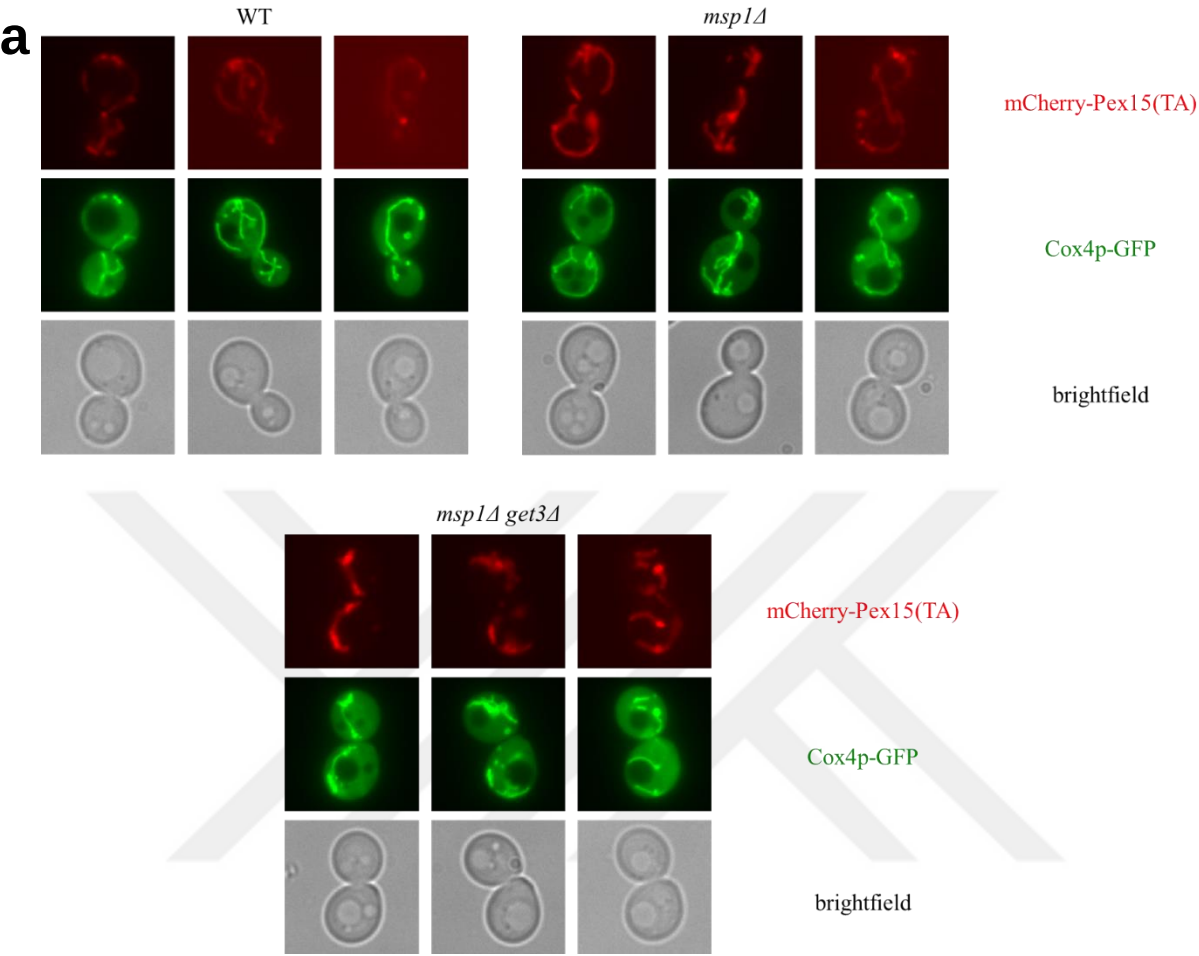


Figure 4.5 mCherry-Pex15p localizes to different intracellular membranes. mCherry-Pex15p targets structures resembling mitochondria in WT cells or ER in *spf1Δ* cells by disrupting peroxisomal biogenesis. WT or *spf1Δ* strains BY4742 or CDD949 were transformed with plasmids expressing peroxisomal matrix-localized sfGFP (b311) and mCherry-Pex15p (b334). Cells were examined by fluorescence microscopy. Scale bar, 5 μ m.

4.2.4 Peroxisomal targeting of mCherry-Pex15(TA) fusion proteins is affected by Msp1p and Get3p

The main pathway that targets TA proteins to ER membrane in *S. cerevisiae* is the GET pathway, which consists of Get3p receptor that recognizes TA proteins destined for the secretory system and recruits them to the GET complex at the ER membrane. In this

pathway, recognition by Get3p seems to be the decisive step between ER and mitochondrial membranes as its deletion causes ER destined TA proteins to mislocalize to mitochondria ⁷¹. Recently, three new proteins, Snd1p, Snd2p, and Snd3p, that may constitute an alternative pathway, which is called the SRP-independent targeting (SND) pathway, for TA proteins at the ER has been suggested ⁶⁸. Remarkably, deleting key components of these two pathways, Get3p or Snd2p, did not alter the peroxisomal distribution of mCherry-YgiM(TA) in *S. cerevisiae* cells, although it is worth noting that Snd2p deletion has not been confirmed (C. Dunn, unpublished results). To investigate whether mCherry-Pex15(TA) would mislocalize to mitochondria in a similar genetic background, we used *msp1Δ get3Δ* strain. We used the double deletion strain to have a more profound effect as Msp1p was also suggested to have a role in preventing mislocalization of ER targeted TA proteins to mitochondria, similar to Get3p ¹⁹⁰. Although we observed mitochondrial mislocalization of mCherry-Pex15(TA) in this genetic background, this phenotype is probably an experimental artifact as mCherry-Pex15(TA) mislocalization to mitochondria is observed also in the wild-type cells (Figure 4.6a), suggesting an already disturbed PMP biogenesis. We also observed peroxisomal localization of mCherry-Pex15(TA) in WT and *msp1Δ* cells while its distribution seems completely mitochondrial in *msp1Δ get3Δ* double mutants. Msp1p has recently been suggested to function in keeping excess TA proteins away from peroxisomal membrane ²⁴¹. Thus, loss of Msp1p, a protein that functions in clearance of mis-targeted or excess TA proteins both from mitochondrial and peroxisomal membranes, might have resulted in elevated localization of mCherry-Pex15(TA) at both membranes, although the relevant quantification was not performed. On the other hand, loss of Get3p in *msp1Δ* genetic background did not affect the mitochondrial localization of the protein while its peroxisomal localization is disrupted probably because of the specific role of GET pathway in processing TA proteins destined for the endomembrane system (Figure 4.6a and b). It is also worth noting that similar to mCherry-Pex15p, expression of mCherry-Pex15(TA) disrupts peroxisomal biogenesis in certain portion of the cells as quantified in the following section (Figure 4.7).



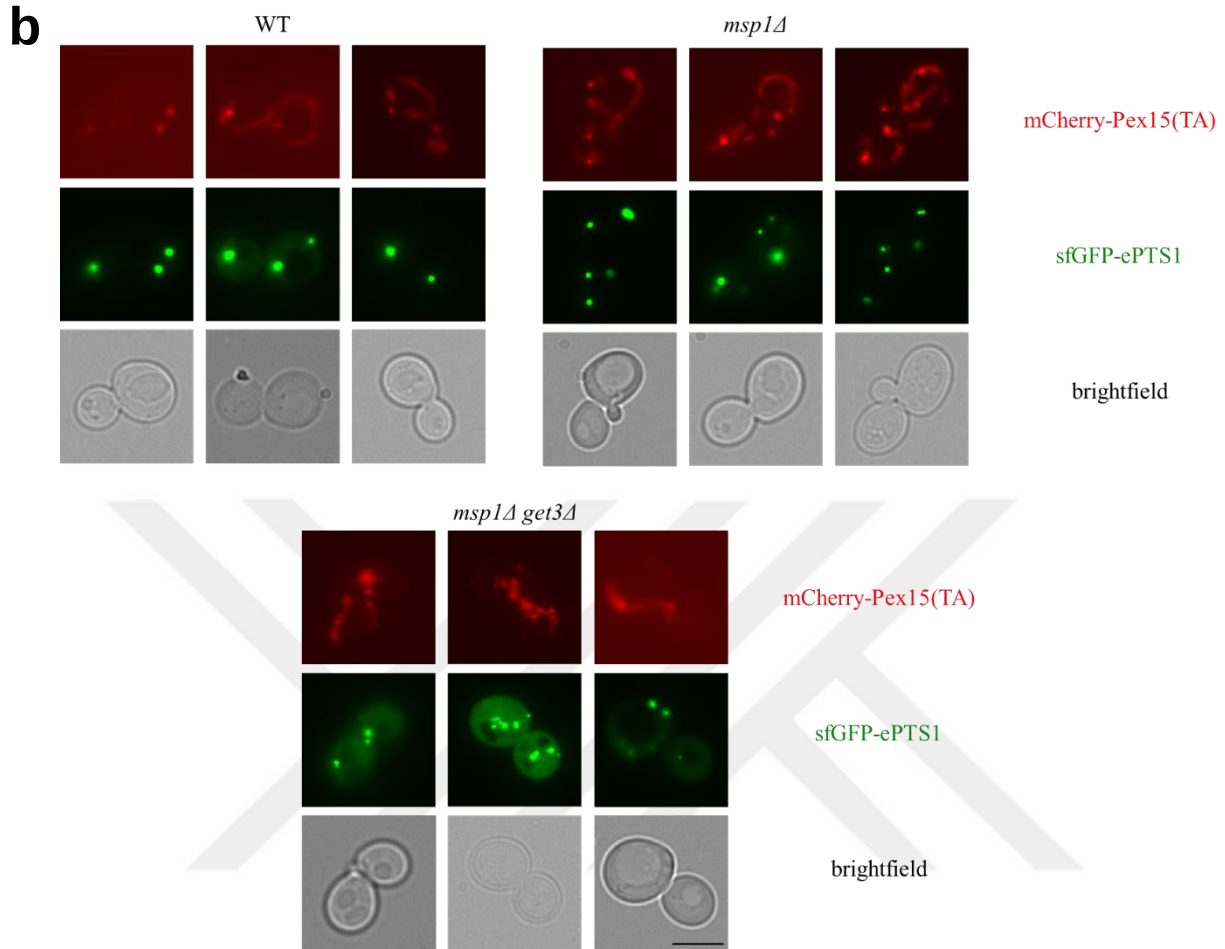


Figure 4.6 mCherry-Pex15(TA) dual-localizes to mitochondria and peroxisomes. **a.** mCherry-Pex15(TA) targets mitochondria in WT, *msp1Δ*, and *msp1Δ get3Δ* cells. WT, *msp1Δ*, or *msp1Δ get3Δ* strains BY4741, CDD1154, or CDD1155 were transformed with plasmids expressing mitochondria-localized Cox4p(1-21)-GFP (pHS1) and mCherry-Pex15(TA) (b365). **b.** mCherry-Pex15(TA) targets peroxisomes in WT, *msp1Δ*, and partially in *msp1Δ get3Δ* cells. Strains in (a) were transformed with plasmids expressing peroxisomal matrix-localized sfGFP (b311) and mCherry-Pex15(TA) (b365). Cells were examined by fluorescence microscopy. Scale bar, 5 μ m.

4.2.5 mCherry-YgiM(TA) fusion protein labels peroxisomal membranes with the least artifact compared to its functional analogues

As indicated by several other studies¹⁶⁹, and observed in our results, overexpression of a native peroxisomal protein, especially if fused to a fluorescent tag, changes peroxisomal distribution and morphology. In order to quantify the level of this change, we separately expressed several mCherry fusion proteins containing different peroxisomal targeting sequences in wild-type cells and assessed the number of mature peroxisomes per cell according to sfGFP-ePTS1 signal. Among four different fusion proteins in which a peroxisomal targeting sequence is fused to the C-terminus of mCherry fluorescent protein, mCherry-Pex15(TA) affected the import of peroxisomal matrix-localized sfGFP most dramatically. On the other hand, the effect of mCherry-YgiM appeared similar to that of vector while cells expressing mCherry-Fis1(TA) or mCherry-ePTS1 caused in between phenotype (Figure 4.7).

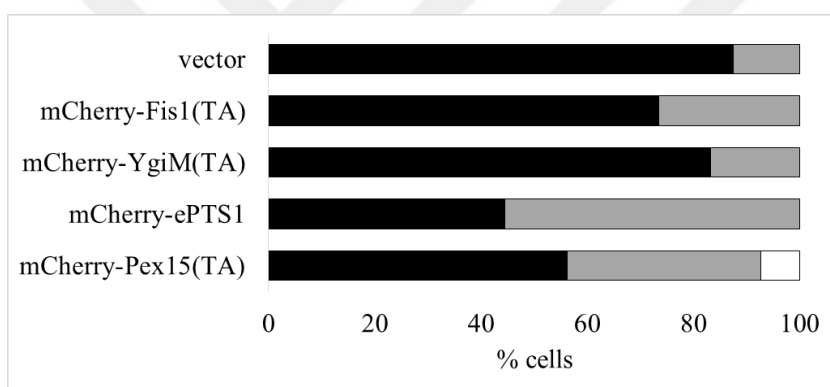


Figure 4.7 Bacterial YgiM TA causes the least artifact on peroxisomes compared to other peroxisome localizing sequences. The YgiM TA can label peroxisomal membranes with the least peroxisomal disruption compared to other targeting sequences that can label peroxisomes when fused to the C-terminus of mCherry fluorescent tag. Wild-type strain BY4742 was transformed with plasmid b311 expressing peroxisomal matrix-localized sfGFP, followed by transformation with plasmids expressing either of the mCherry-Fis1(TA) (b109), mCherry-YgiM(TA) (b274), mCherry-ePTS1 (b364), mCherry-Pex15(TA) (b365) peroxisome-localized fusion proteins or with empty vector pRS315. Quantification of peroxisomal morphology of the transformants was performed blind to genotype. White bar represents cells lacking import competent peroxisomes, grey bar represents cells with import competent peroxisomes, but competent to a less extent than wild-type cells, and black bar represents cells with import competent peroxisomes.

Quantification was repeated three times ($n > 200$ per genotype), and a representative experiment is shown.

4.3 Discussion

Our results, in which a tail anchor domain obtained from an *E. coli* protein can target and function in peroxisomes enabled us to develop a tool to study the trafficking of peroxisomal membranes within the yeast endomembrane system. The fact that it is able to localize mature peroxisomes or ER-associated peroxisomal membranes in different *S. cerevisiae* mutants allows visualization of peroxisomal trafficking of tail-anchored proteins. Furthermore, unlike other fusion proteins used to label peroxisomal membranes^{169,194}, it does not interfere with the peroxisomal morphology, distribution, and protein biogenesis, providing a preferred experimental environment with less artifact.

As in the case of YgiM TA, presence of a negatively charged amino acid and a longer TMD compared to MOM destined TA proteins are evocative of insertion at ER membranes⁵⁶. Interestingly, probability of YgiM TA to form transmembrane helix is quite higher compared to Pex15p and PEX26 (Figure 4.2) tail-anchored proteins that are known to interact with Pex3 and Pex19 during direct peroxisomal targeting and the TA of YgiM do not resemble that of Pex15p and PEX26. In fact, the last 50 and 30 amino acids found at the C-terminus of PEX26 and Pex15p, respectively, are suggested to be Pex19 binding sites¹⁹¹. Thus, this structural and sequential difference might be indicative of YgiM TA not interacting with the components of direct peroxisomal import pathway. An organelle marker that does not interfere with the intrinsic import pathway of the organelle would be a preferred characteristic. It is also important to note that structural characteristic of YgiM TA resembles that of Fis1p and ER-localized cytochrome b5, that are suggested to spontaneously insert at mitochondrial⁸⁵ and ER membranes⁷⁵.

Our analyses with different yeast mutant strains suggest that YgiM TA does not follow classical pathways of ER destined TA proteins on its route to peroxisomes or there might be redundant pathways for its peroxisomal targeting. YgiM TA seems to reach

peroxisomes independently of the conserved GET pathway (C. Dunn, unpublished results) that specifically interacts with ER-destined TA proteins ⁷¹. In fact, this interaction is suggested to have a role in tail-anchored Pex15p to reach mature peroxisomes ⁶², although *get3Δ* yeast cells do have peroxisomes ^{62,71}, unlike Pex15p deficient cells. The recently discovered SND pathway that is proposed to be alternative to GET pathway does not seem to have a role in YgiM targeting to peroxisomes either (C. Dunn, unpublished results), although the localization of YgiM TA in a strain in which both of these redundant pathways are perturbed remains to be investigated. According to our results, mCherry tagging of the native peroxisomal protein Pex15p does not provide reliable imaging in the mutants of yeast TA protein pathway as distribution of mCherry-Pex15p fusion protein and peroxisomal biogenesis is already disturbed in the wild-type cells, although the extent of either of this disruptions is not quantified. Similarly, mCherry-Pex15(TA) fusion protein, disrupts peroxisomal biogenesis in the wild-type cells. Alternatively, YgiM TA, which causes such artifacts minimally, with an mCherry fluorescent tag on its N-terminus can be used to visualize mature peroxisomes in different yeast mutants.

Although often suggested to lack any peroxisomal membrane ¹⁶², *pex3Δ* or *pex19Δ* mutants were discovered to possess immature peroxisomal vesicles that are able to recruit some of the PMPs ¹¹⁷. Similarly, YgiM TA appears to localize to immature peroxisomal vesicles in the same genetic background (C. Dunn, unpublished results). Here, we show that these peroxisomal vesicles seem to be near ER membranes suggesting that they are indeed intermediate membranes on ER to mature peroxisome route, although the association between these two structures should be confirmed with high-resolution microscopy techniques. Therefore, like native PMPs, bacterial YgiM TA can also be used to label immature peroxisomes.

If bacterial YgiM TA does not follow the standard TA machineries of ER membrane that would eventually insert the protein at peroxisomal vesicles, what is the mechanism that allows its peroxisomal localization? It is often suggested that PMPs follow redundant pathways, either following an ER to peroxisome route (indirect pathway) or inserting at peroxisomal membranes from the cytosol (direct pathway), a process that requires Pex19p to recruit cargo proteins at membrane bound Pex3p ¹⁶⁷. However, insertion of YgiM TA does not seem to depend only on GET or SND pathways and is able to insert at Pex14p containing

vesicles in *pex3Δ* or *pex19Δ* mutants, although *snd2Δ* mutant strains that were used in these analyses need confirmation. On the other hand, it is unlikely that YgiM TA follows the direct pathway, because of the difference in its amino acid sequence compared to PEX26 and Pex15p proteins, a protein characteristic suggested to be necessary for Pex19 interaction¹⁹¹. One possibility is that YgiM insertion at peroxisomal membranes follow redundant SND or GET pathways, similar to most other PMPs¹¹⁷, considering the fact that localization of YgiM TA in a *get3Δ snd2Δ* has not been tested.

Alternatively, insertion of YgiM TA to peroxisomal membranes might be independent of any of the known membrane insertion machineries. Indeed, it is not extraordinary for TA proteins to insert biological membranes without the assistance of any cellular factors. There are proteins that can insert at ER⁴⁹ or mitochondrial membranes⁴⁸, independent of the membrane specific translocators. Membrane insertion of these proteins was suggested to be dependent on a unique TMD⁷⁴, or on the ergosterol content of the target membrane⁸⁵. However, the TMD characteristics of YgiM TA do not quite resemble ER TA proteins that are capable of unassisted insertion at ER membranes such as cytochrome b5⁴⁹ (Figure 4.1). On the other hand, it is worth noting that insertion of YgiM TA seems to be also affected by the ergosterol content of the target membrane, a phenomenon that is observed in Fis1p that inserts at both mitochondrial and peroxisomal membranes⁸³ and is suggested to be a spontaneously inserting TA protein⁸⁵. Nevertheless, regardless of its insertion machinery, YgiM TA could serve a valuable tool to track mature and immature peroxisomal membranes.

It is also important to address the significance of peroxisomal localization of a bacterial TA from an evolutionary perspective. By the time peroxisomes were first discovered²⁴² and described as an organelle²³⁷, there had been speculations about their evolutionary origin. These speculations include peroxisomes being originated from the outer membrane vesicles released by the mitochondrial ancestor²⁰², or being originated through endosymbiosis similar to mitochondria and plastids²²⁰. Our findings, in which a bacteria-derived TA targets peroxisomal membrane, are not against either of these hypotheses. If the eukaryotic endomembrane system, including peroxisomes, has been originated from mitochondrial-derived vesicles containing lipids of a prokaryotic endosymbiont, then it is not surprising for the TA of a bacterial plasma membrane protein YgiM²⁴³ localizing to the

peroxisomal membrane. The fact that peroxisomal proteins often mistarget mitochondria in the absence of Msp1p surveillance¹⁹⁰ or dual targeting of tail-anchored Fis1p to mitochondria and peroxisomes⁸³, suggesting resemblance of the two membrane, are also consistent with this view. Alternatively if peroxisomes have evolved from an anaerobic hydrogen-producing prokaryote^{220,244}, it is expected that the peroxisomal membrane should again resemble prokaryotic membranes. Thus, localization of the bacterial plasma membrane protein YgiM TA to peroxisomal membrane is not evidence against this view either.

One might wonder the purpose of using a prokaryotic protein to visualize peroxisomes while there are several different membrane or matrix destined peroxisomal proteins or targeting sequences that can be used to visualize immature and mature peroxisomes. Although fluorescently tagged PTS1 or PTS2 are generally used to label peroxisomes²⁴⁵, they are the peroxisomal targeting signals of most of the matrix proteins¹²³ and can only label import competent peroxisomes at the very mature stage, not the peroxisomal membrane ghosts that cannot import matrix proteins⁸⁴. Thus, these fusion proteins can only label peroxisomes in wild-type strains and cannot be used to follow early peroxisomal membranes. Although these early membranes were reported to possess some of the PMPs^{108,117}, and peroxisomal targeting with fluorescently tagged PMPs has been represented²⁴⁵, use of such fusion proteins is not recommended as puncta distribution of these proteins may not always reflect correct targeting to peroxisomal membranes. One example of such phenotype is also demonstrated here in this study with mCherry-Pex15(TA) mislocalization to the MOM in the wild-type strains. Furthermore, puncta distribution of these proteins might be the result of mistargeting to other intracellular membranes¹⁹⁴, as overexpression of fluorescently tagged PMP fusions may have unexpected effects on peroxisomal biogenesis, changing their abundance and morphology¹⁶⁹, while expressing such fusion proteins under their native promoter might result in very weak fluorescent signal. On the other hand, YgiM itself is not a protein involved in peroxisomal biogenesis, unlike PMP targeting signals PTS1 and PTS2, or the TA of Pex15p. Thus, mCherry-YgiM(TA) appears to disrupt peroxisomal biogenesis similar to an empty vector, unlike other fusion proteins containing a peroxisome localizing sequence. Thus, we suggest that mCherry-YgiM(TA) fusion protein derived from *E. coli* genome could serve as a functional tool for peroxisomal trafficking studies in different *S. cerevisiae* mutants. Furthermore, YgiM TA

seems to localize peroxisomal membranes also in mammalian cells (B. Seferoğlu, unpublished results). Whether it can be used for peroxisomal labeling also in mammalian cells will be further investigated.

4.4 Conclusion

This work demonstrates that a TA domain obtained from bacterial proteins can insert and function at peroxisomal membranes. According to these results, we suggest a tool to follow the intracellular trafficking of peroxisomes, the TA of bacterial protein YgiM. This protein domain can localize to peroxisomal vesicles on their route from the ER to mature peroxisomes. Furthermore, as YgiM TA is not an intrinsic protein sequence that functions in peroxisomes, it does not interfere with peroxisomal biogenesis, changing their morphology and distribution when fused to a fluorescent tag, unlike most other peroxisomal proteins or targeting sequences. Thus, we propose that bacterial YgiM TA can serve as an alternative tool to study the intracellular trafficking of peroxisomes.

4.5 Materials and Methods

4.5.1 Yeast strains and plasmids

Details and associated references of strains used in this study are given in Table 4.1. Construction details of plasmids and related references of the acquired plasmids are provided in Table 4.2. Oligonucleotides utilized in this work are listed in Table 4.3.

4.5.2 Media and culture conditions

Synthetic complete (SC) medium consists of 0.67% yeast nitrogen base lacking amino acids, 0.1% casamino acids, 2% dextrose, 50 µg/ml adenine hemisulfate, and either 100 µg/ml L-tryptophan (SC-Ura) or 25 µg/ml uracil (SC-Trp). Supplemental minimal medium (SMM) consists of 0.67% yeast nitrogen base lacking amino acids, 2% dextrose, 20 µg/ml adenine hemisulfate, 20 µg/ml methionine, 20 µg/ml uracil, 30 µg/ml lysine. SMM also contains 20 µg/ml histidine, 100 µg/ml leucine, and/or 20 µg/ml tryptophan depending on selection condition. Solid media also contain 1.7% bacteriological agar. Cells were incubated at 30°C unless indicated otherwise.

4.5.3 Microscopy

For epifluorescence microscopy studies, cells grown to logarithmic phase of proliferation were examined by using an Eclipse 80i microscope with a 100X Plan Fluor objective and linked to a DS-Qi1Mc camera (Nikon, Tokyo, Japan). Cells were grown in SC or SMM medium suitable for plasmid selection. Images were captured using NIS-Elements version of AR 3.2 and exposure times were set automatically. mCherry fusions are driven by the ADH1 promoter and universally contain Fis1p amino acids 119-128 (not necessary or sufficient for mitochondrial targeting) linking mCherry to YgiM TA. Microscopic assessment of Pex15p variant functionality was performed by scoring peroxisomal morphology blind to genotype.

Table 4.1 Strains used in Chapter 4

Strain	Genotype	Source	Parental Strain	Method Used
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	EUROSCARF Y00000		
BY4742	<i>MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0</i>	EUROSCARF Y10000		

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RJ867	<i>MATa leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 trp1Δ63</i>	246		
CDD51	<i>MATa ura3-1 trp1Δ2 ade2- 1 leu2-3,112 his3-11,15 can1-100 cyh2</i>	247		
CDD65	<i>MATa his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0 cyh2</i>	248		
CDD463	<i>MATa leu2Δ0 his3Δ200 met15Δ0 trp1Δ63 ura3Δ0</i>	229		
CDD898	<i>MATa/α leu2Δ0/leu2Δ0 his3Δ200/his3Δ200 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 ura3Δ0/ura3Δ0</i>	94		
CDD949	<i>MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 yel031wΔ::kanMX4</i>	EUROSCARF Y10272		
CDD953	<i>MATα ura3Δ0 leu2Δ0; his3Δ1 lys2Δ0 ydl100cΔ::kanMX4</i>	EUROSCARF Y13797		
CDD974	<i>MATα ura3Δ0 leu2Δ0; his3Δ1 lys2Δ0 ydr329cΔ::kanMX4</i>	EUROSCARF Y13688		
CDD975	<i>MATα ura3Δ0 leu2Δ0 his3Δ1 lys2Δ0 ydl065cΔ::kanMX4</i>	EUROSCARF Y13762		

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CDD993	<i>MATa/α his3Δ1/HIS3</i> <i>leu2Δ0/LEU2</i> <i>met15Δ0/MET15</i> <i>ura3Δ0/URA3</i> <i>YDL100/ydl100cΔ::KanMX4</i> <i>PEX30/pex30Δ::hphNT1</i>	constructed by Dr. C. Dunn	BY4741, CDD953	BY4741 was mated to CDD953. In resulting diploids, <i>PEX30</i> was disrupted with <i>hphNT1</i> using primers 1006/1007 and template b80
CDD1012	<i>MATa/α his3Δ1/ his3Δ1</i> <i>leu2Δ0/leu2Δ0</i> <i>MET15/met15Δ0</i> <i>LYS2/lys2Δ0</i> <i>ura3Δ0/ura3Δ0</i> <i>MSP1/msp1Δ::kanMX4</i>	94		
CDD1034	<i>MATa LEU2 MET15</i> <i>ura3Δ0</i> <i>YDL100/YDL100</i> <i>pex30Δ::URA3</i>	constructed by Dr. C. Dunn	CDD993	CDD993 was sporulated, and <i>hphNT1</i> was replaced with <i>URA3</i> using primers 1006/1007 and template pRS306.
CDD1043	<i>MATa/α his3Δ1/ his3Δ1</i> <i>leu2Δ0/leu2Δ0</i> <i>met15Δ0/MET15</i> <i>LYS2/lys2Δ0</i> <i>ura3Δ0/ura3Δ0</i> <i>MSP1/msp1Δ::kanMX4</i> <i>GET3/get3Δ::hphNT1</i>	constructed by Dr. C. Dunn	CDD1012	<i>GET3</i> was disrupted with <i>hphNT1</i> using primers 1051/1052 and template b80
CDD1046	<i>MATa/α leu2Δ0/LEU</i> <i>MET15/met15Δ0</i> <i>LYS2/lys2Δ0</i> <i>MSP1/msp1Δ::kanMX4</i> <i>GET3/get3Δ::hphNT1</i> <i>PEX30/pex30Δ::URA3</i>	constructed by Dr. C. Dunn	CDD1034 , CDD1043	CDD1043 was sporulated, and a G418-resistant and hygromycin-resistant spore was mated to CDD1034
CDD1140	<i>MATa/MATa</i> <i>ura3Δ0/ura3Δ0</i>	EUROSCARF Y31735		

	<i>leu2Δ0/leu2Δ0</i> <i>his3Δ1/his3Δ1</i> <i>met15Δ0/MET15</i> <i>LYS2/lys2Δ0</i> <i>yol044wΔ::kanMX4/yol044wΔ::kanMX4</i>			
CDD1154	<i>MET15 lys2Δ0 ura3Δ0</i> <i>msp1Δ::kanMX4</i>	constructed by Dr. C. Dunn	CDD1046	Sporulation
CDD1155	<i>MET15 lys2Δ0 ura3Δ0</i> <i>msp1Δ::kanMX4</i> <i>get3Δ::hphNT1</i>	constructed by Dr. C. Dunn	CDD1046	Sporulation
CDD1171	<i>MATa/α</i> <i>yol044wΔ::kanMX4/yol044wΔ::kanMX4</i> <i>trp1Δ63/ trp1Δ63</i> <i>ura3Δ0/ura3Δ0</i> <i>leu2Δ0/leu2Δ0</i> <i>his3Δ1/his3Δ1</i> <i>met15Δ0/ met15Δ0</i> <i>LYS2/LYS2 cyh2/cyh2</i> +plasmid b311 [pHIS3- sfGFP-ePTS1] +plasmid b340 [p TRP1- CYH2-mCherry-PEX15]	constructed by Dr. C. Dunn	CDD65, CDD1140	CDD1140 was sporulated and mated to CDD65. The resulting diploids were sporulated, selected on 10 mg/L CHX, spores of opposite mating type were transformed with b311 or b340 and mated.
CDD1182	<i>MATa/α</i> <i>yol044wΔ::kanMX4/yol044wΔ::kanMX4</i> <i>trp1Δ63/ trp1Δ63</i>	constructed by Dr. C. Dunn	CDD1171	CDD1171 was transformed with b326, counter-selected for b340, transformed with b354, and cured of b326.

	<i>ura3Δ0/ura3Δ0</i> <i>leu2Δ0/leu2Δ0</i> <i>his3Δ1/his3Δ1</i> <i>met15Δ0/ met15Δ0</i> <i>LYS2/LYS2 cyh2/cyh2</i> +plasmid b311 [pHIS3- <i>sfGFP-ePTS1</i>] +plasmid b354 [pTRP1- <i>CYH2-PEX15(1-331)-</i> <i>PEX15(TA)</i>]			
CDD1200	<i>MATα his3Δ1 leu2Δ0</i> <i>lys2Δ0 ura3Δ0 PEX14-</i> <i>sfGFP::SpHIS5</i>	constructed by Dr. C. Dunn	BY4742	PEX14 was tagged with sfGFP using primers 1370/1371 and template b93
CDD1201	<i>MATα ydr329cΔ::kanMX4</i> <i>PEX14-sfGFP::SpHIS5</i>	constructed by Dr. C. Dunn	CDD974	PEX14 was tagged with sfGFP using primers 1370/1371 and template b93.
CDD1202	<i>MATα ydl065cΔ::kanMX4</i> <i>PEX14-sfGFP::SpHIS5</i>	constructed by Dr. C. Dunn	CDD975	PEX14 was tagged with sfGFP using primers 1370/1371 and template b93.

Table 4.2 Plasmids used in Chapter 4

Plasmid	Name	Source	Construction details or source
pRS313	pARS-CEN-HIS3	231	

pRS314	pARS-CEN-TRP1	231	
pRS315	pARS-CEN-LEU2	231	
pRS316	pARS-CEN-URA3	231	
pRS306	pARS-URA3	231	
pHS1	pCOX4(1-21)-GFP-HIS3	249	
pKS1	pTRP1-CYH2	233	
b80	pRS306H	234	

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b93	pFA6a-link- yoSuperfolderGFP-SpHis5	235	
b100	pCYH2-TRP1-GAL4- sfGFP-FIS1	94	
b109	pLEU2-mCherry-FIS1(TA)	94	
b151	pHIS3-mCherry-FIS1(TA)	constructed by A. Keskin	The insert of plasmid b109 was amplified using primers 11 and 12. The PCR product was co-transformed with <i>NotI</i> -cut pRS313 into strain CDD463.
b152	pTRP1-mCherry-FIS1(TA)	constructed by A. Keskin	The insert of plasmid b109 was amplified using primers 11 and 12. The PCR product was co-transformed with <i>NotI</i> -cut pRS314 into strain CDD463.
b212	pSM1959	250	pSM1959 was a gift from Susan Michaelis (Addgene plasmid # 41837)
b238	pCYH2-TRP1-FIS1p	94	

b252	pLEU2-mCherry- Δ TA	94	
b274	pLEU2-mCherry-ygiM(TA)	constructed by A. Keskin	The <i>ADH1</i> promoter and mCherry was amplified from plasmid b152 using primers 11 and 764 The region encoding the predicted tail anchor of YgiM was amplified from <i>Escherichia coli</i> strain DH5 α using primers 963 and 964. The <i>ADH1</i> 3' UTR was amplified from plasmid b152 using primers 775 and 12. PCR products were co-transformed with <i>NotI</i> -cut pRS315 into strain BY4743.
b311	pHIS3-sfGFP-ePTS1	This study	The <i>ADH1</i> promoter was amplified from plasmid b109 ⁹⁴ using primers 11 and 1158. SuperfolderGFP was amplified from plasmid b100 ⁹⁴ using primers 1110 and 1111. The <i>ADH1</i> 3'UTR was amplified from plasmid b109 using primers 775 and 12. The enhanced ²⁵¹ PTS1 sequence was generated by annealing primers 1275 and 1276 and performing one round of PCR extension. PCR products were co-transformed with <i>NotI</i> -cut pRS313 into strain CDD898.
b326	pURA3-PEX15(1-331)- PEX15(TA)	constructed by Dr. C. Dunn and G. Lutfullahoğlu Bal	The <i>PEX15</i> ORF lacking the portion encoding the tail anchor was amplified from <i>Saccharomyces cerevisiae</i> gDNA using primers 1303 and 1306. The Pex15p TA was amplified from <i>Saccharomyces cerevisiae</i> gDNA using primers 1304 and 1305. The <i>ADH1</i> 3'UTR was amplified from plasmid b109 using primers 775 and 12. PCR products were co-transformed with <i>NotI</i> –cut pRS316 into competent <i>Saccharomyces cerevisiae</i> cells.

b329	<i>pURA3-PEX15(1-331)-ygiM(TA)</i>	constructed by Dr. C. Dunn and G. Lutfullahoğlu Bal	The <i>PEX15</i> ORF lacking the portion encoding the tail anchor was amplified from <i>Saccharomyces cerevisiae</i> gDNA using primers 1303 and 1306. The TA of bacterial protein YgiM was amplified from plasmid b274 using primers 1048 and 12. PCR products were co-transformed with <i>NotI</i> –cut pRS316 into competent <i>Saccharomyces cerevisiae</i> cells.
b330	<i>pURA3-PEX15(1-331)-yqiD(TA)</i>	constructed by Dr. C. Dunn and G. Lutfullahoğlu Bal	The <i>PEX15</i> ORF lacking the portion encoding the tail anchor was amplified from <i>Saccharomyces cerevisiae</i> gDNA using primers 1303 and 1306. The TA of bacterial protein YqiD was amplified from plasmid b274 using primers 1048 and 12. PCR products were co-transformed with <i>NotI</i> –cut pRS316 into competent <i>Saccharomyces cerevisiae</i> cells.
b334	<i>pURA3-mCherry-PEX15</i>	This study	The <i>ADH1</i> promoter and mCherry was amplified from plasmid b109 ⁹⁴ using primers 11 and 1158. The <i>PEX15</i> ORF was amplified from gDNA (CDD48) using primers 1317 and 1322. The <i>ADH1</i> 3'UTR was amplified from plasmid b109 using primers 1321 and 12. PCR products were co-transformed with <i>NotI</i> -cut pRS316 into strain CDD898.
b340	<i>pTRP1-CYH2-mCherry-PEX15</i>	constructed by Dr. C. Dunn	The insert of plasmid b334 was amplified using primers 11 and 12. The PCR product was co-transformed with <i>NotI</i> -cut pRS314 into strain CDD898.
b341	<i>pURA3-PEX15(1-331)</i>	This study	The <i>PEX15</i> promoter and the <i>PEX15</i> ORF lacking the portion encoding the tail anchor was amplified from gDNA (CDD993) using primers 1303 and 1306. The <i>ADH1</i> 3'UTR was amplified from plasmid b252 using primers 1048 and 12. PCR products were co-

			transformed with <i>NotI</i> -cut pRS316 into strain CDD898.
b354	<i>pTRP1-PEX15(1-331)-PEX15(TA)</i>	This study	The insert of plasmid b326 was amplified using primers 11 and 12. The PCR product was co-transformed with <i>NotI</i> -cut pKS1 into strain CDD463.
b364	<i>pLEU2-mCherry-ePTS1</i>	This study	The <i>ADH1</i> promoter and mCherry was amplified from plasmid b151 using primers 1048 and 12. The enhanced ²⁵¹ PTS1 sequence was amplified from plasmid b311 using primers 764 and 11. PCR products were co-transformed with <i>NotI</i> -cut pRS315 into strain CDD463.
b365	<i>pLEU2-mCherry-PEX15(TA)</i>	This study	The <i>ADH1</i> promoter and mCherry was amplified from plasmid b152 using primers 1048 and 12. The Pex15p TA and the <i>ADH1</i> 3'UTR was amplified from plasmid b326 using primers 1304 and 1305. PCR products were co-transformed with <i>NotI</i> -cut pRS315 into strain CDD898.

Table 4.3 Oligonucleotides used in Chapter 4

Name	Sequence (5'-3')
11	GTAAAACGACGGCCAGTGAATTG
12	CGCCAAGCTCGGAATTAACCCTCAC
764	TGTTTCCTTCTGGATCTTATCCTCTACCATCTTGTACAGCTCGTCCATGCCGCCGGTGGA
775	TAAACACTTCTAAATAAGCGAATTTCTTATGATTTATGAT
963	ATGGTAGAGGATAAGATCCAGAAGGAAACATGGTTTATGTATGGCGGTGGCGTGCTGGG G
964	ATCATAAGAAATTCGCTTATTTAGAAGTGTTTAGTTCATCCAGCGATCTTTGCGTTTGCG

1006	CTGTCTAGTTGATCCTCCGGAGTGTA AAAA ACTGATTTTCAAGATTGTACTGAGAGTGCAC
1007	TAGAGATTATATTATGTAAAGGTAAAAACGGGAGCGAGCACTGTGCGGTATTTACACCG
1048	ATGGTAGAGGATAAGATCCAGAAGGAAACA
1051	AAACGTACGACAAGAACAAGAAGATCATCACATTGTAATTAGATTGTACTGAGAGTGCA C
1052	TTATATGTCGTATGTATCTATTTATGGTATTCAGGGGCTTCTGTGCGGTATTTACACCG
1110	CCAAGCATACAATCAACTATCTCATATACAATGTCTAAAGGCGAGGAATTGTTTACAGGT
1111	TGTTTCCTTCTGGATCTTATCCTCTACCATTTTGTACAATTCGTCCATTCTAATGTTAT
1158	TGTATATGAGATAGTTGATTGTATGCTTGG
1275	ATGGTAGAGGATAAGATCCAGAAGGAAACACTGGGGCGTGGCAGAGAAGTAAGCTAT GA
1276	ATCATAAGAAATTCGCTTATTTAGAAGTGTTTCATAGCTTACTTCTTCTGCCACGCCCCAG
1303	TGTTTCCTTCTGGATCTTATCCTCTACCATACTTCTTGTGAAATGATGCTTCAGG
1304	ATGGTAGAGGATAAGATCCAGAAGGAAACAGTGCTGAACAAAAACGGACTTCTG
1305	ATCATAAGAAATTCGCTTATTTAGAAGTGTTTATATACTCGCTAGAAGTTTTAGCAACCC
1306	GACTCACTATAGGGCGAATTGGAGCTCCACTTTTCCGTACTCTCCAAGATAAAGGGAAAG
1317	TCCACCGGCGGCATGGACGAGCTGTACAAGGCTGCAAGTGAGATAATGAACAATCTGCC T
1321	ATCATAAGAAATTCGCTTATTTAGAAGTGTTCACTTATATTTTTTCAAACATAAGAGTAG
1322	GGGTTGCTAAAACCTTCTAGCGAGTATATGAACACTTCTAAATAAGCGAATTTCTTATGAT
1370	CCCTGACTGGCAAAATGGACAGGTCTGAAGACTCCATCCCAGGTGACGGTGCTGGTTTA
1371	CAATTTCCGTTAAAAAACTAATTACTTACATAGAATTGCGTCGATGAATTCGAGCTCG

Chapter 5

A METHOD TO INVESTIGATE MITOCHONDRIAL IMPORT COMPONENTS IN MAMMALIAN SYSTEMS

5.1 Introduction

Mitochondria function in a number of key events in the eukaryotic cell. Being responsible for ATP production²⁵², it functions as the main energy production center of the cell, take a role in biogenesis of Fe-S clustered proteins²⁵³, and helps to decide cellular fate by activating apoptosis²⁵⁴. Mitochondrial dysfunction is known to be related with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease²⁵⁵. As mitochondria is decisive for apoptosis and cellular fate, mitochondrial dysfunction is also suggested to cause cancer and tumorigenesis²⁵⁶. Having 99% of its proteins imported from the cytosol, proper mitochondrial protein import is clearly essential for proper mitochondrial functioning^{257,258}. Mutations in mitochondrial import machinery in particular, are related with genetic disorders including human deafness dystonia syndrome, dilated cardiomyopathy with ataxia, and spastic paraplegia²⁵⁸. Therefore, a clear understanding of mitochondrial import in human cells is critical for having a precise approach towards diseases related with mitochondrial dysfunction.

Targeting of protein precursors to mitochondria requires the presence of signal sequences called presequences that are mostly found in the N-terminus of the precursor protein³⁴. The receptors found in the outer membrane of mitochondria Tom20 and Tom70 are the initial docking sites for protein precursors, and most of the precursor proteins enter mitochondria through a common gate, called TOM complex. After being translocated across the outer membrane the proteins may follow different routes depending on their final destination.

Intermembrane space resident proteins that contain cysteine-rich signals are recruited to the mitochondrial intermembrane space import and assembly (MIA) machinery that drives protein import into the intermembrane space. Outer or inner membrane integral proteins are recruited by the small Tim chaperones found in the intermembrane space to the relevant membrane. Sorting and assembly machinery (SAM) complex guides β -barrel proteins to be integrated to the outer membrane while direct passage to translocase of the inner membrane 22 (TIM22) results in lateral sorting of the metabolite carrier proteins to the inner membrane, a process that driven by the membrane potential. Lastly, proteins with a presequence directly pass to the translocase of the inner membrane 23 (TIM23) complex. At TIM23, proteins are either laterally sorted to the inner mitochondrial membrane in a membrane potential dependent manner, or imported into the matrix with the help of ATP-driven presequence translocase-associated import motor (PAM). Upon translocation to the matrix, presequence is cleaved by the mitochondrial processing peptidase ³⁷.

Much of the information regarding protein import in mitochondria has come from studies using fungal species as a model system. Although many of the components identified in fungal systems also have counterparts in mammalian cells and the structure of the core components and events that take place during protein import are quite similar, additional components of ambiguous function have been discovered in mammalian systems ²⁵⁹. Mammalian translation system and cytosol are generally used for *in vitro* mitochondrial import assays. In other words, cytosolic factors having a role in mitochondrial protein import are mostly isolated from mammalian sources while studying protein import in mitochondria, even if the mitochondria is of fungal source ²⁶⁰. Thus, cytosolic factors of mammalian mitochondrial import are relatively better studied. Components such as presequence binding factor (PBF) that was reported to bind specifically to the precursor form of rat ornithine carbamoyltransferase (OTC) ²⁶¹ or mitochondrial import stimulation factor (MSF) proposed to recruit precursor proteins to the outer membrane receptor, TOM70 ²⁶² do not have known functional homologues in lower eukaryotes ²⁵⁹. The TOM complex, the main gate of the outer membrane at which protein translocation starts, has conserved components such as receptors Tom20, Tom22, Tom70, and the channel protein Tom40 that are found in yeast and higher eukaryotes. Although mammalian TOM20 is smaller than its fungal homologue with its 16.3kDa size, it can complement loss of Tom20p and interact with other TOM subunits in yeast ^{263,264}. α -helices in receptor Tom20p mediate an interaction with the

hydrophobic face of presequence of the mitochondrial protein ²⁶⁵. Tom20p was also suggested to interact with the translated mitochondrial presequence to mediate mRNA localization to mitochondria ²⁶⁶. This association mode is also conserved in flies and mammals ²⁶⁷ with an additional component in act, the outer membrane kinase PINK1 that was reported to directly interact with mRNA to recruit it to the MOM ²⁶⁸. Both mammalian ²⁶⁹ and fungal ²⁷⁰ Tom20 are suggested to have function in the maintenance of mitochondrial morphology. Like fungal Tom22p, mammalian TOM22 has C-terminal intermembrane space domain and strongly acidic N-terminal cytosolic domain that interacts with mitochondrial precursor proteins as a receptor. In mammals, the C-terminal intermembrane space domain of TOM22 lacks the acidic character of its fungal counterpart. Although they both function as a protein binding site on the intermembrane side of the MOM, N-terminal segment of yeast Tom22p was shown to interact with presequence of the translocating protein ²⁷¹ while that of human TOM22 was suggested to be required for binding to the mature portion, and no interaction with the preprotein was reported ²⁷². In addition to these large protein members, smaller molecular weight proteins of 10, 7.5, and 5 kDa are found to be associated with mammalian TOM40, suggesting that may represent homologues of yeast Tom7p, Tom6p, and Tom5p ²⁷³. At the inner membrane of mitochondria, subunits of the TIM23 complex, Tim23p, Tim17p, and Tim44p are also present in mammals. While yeast has one Tim17p, an essential component for protein translocation through TIM23 complex ²⁷⁴, mammalian cells possess two homologs TIM17A and TIM17B ²⁷⁵, among which TIM17B seems to have more critical role and TIM17A is degraded under stress conditions ²⁷⁶. Tim44p component of the TIM23 complex is anchored to the matrix face of the yeast mitochondrial inner membrane ²⁷⁷. Such an association is not found in mammalian mitochondria, in which TIM44 is largely soluble in the matrix fraction and do not interact with the TIM23 and TIM17 components at the inner membrane ²⁷⁵. Mammalian TIM44 was suggested to interact with the matrix heat shock protein Hsp70 (mtHsp70) in the absence of ATP ²⁷⁸, whereas yeast Tim44p provide interaction between the translocating protein and the ATPase domain of mtHsp70 to assist protein exit through TIM23 complex ²⁷⁹. The TIM22 complex that functions in the import of integral inner membrane proteins is composed of Tim18p, Tim22p, and Tim54p subunits. While Tim22p has a functional human homologue ²⁸⁰, Tim18p and Tim54p have no identified homologs in mammals ²⁸¹. On the other hand, a recent mammalian study revealed a TIM22 subunit that has no identified

homolog in yeast. TIM29 subunit is required for the protein translocation by maintaining structural integrity of the complex. It was found to be conserved in metazoans with no fungal counterpart²⁸¹. Smaller Tim chaperones that assist protein recruitment from TOM complex to TIM22 complex across the inner membrane including Tim8a, Tim8b (DDP2), Tim9, Tim10a, Tim10b, and Tim13 are conserved between mammals and yeasts with homologous function²⁵⁹. Chaperones found in the matrix space maintain processing, folding, and assembly of the imported mitochondrial proteins. Some of the human chaperones such as MIP catalyzing the maturation of iron-utilizing proteins²⁸², TID regulating the activity of HSP70, HSP60 that is involved in the folding of medium-chain acyl-CoA dehydrogenase variants²⁸³, and HSP10 that assists HSP60²⁸⁴ have identified functions homologous to yeast. On the other hand, functions of the human chaperones mtHsp70 that assists protein import into and/or folding in the matrix.²⁸⁵ and mtGrpE that acts as a nucleotide exchange factor for HSP70²⁸⁶ are inferred from its fungal and mammalian orthologues although they are identified in human mitochondria²⁵⁸.

Mitochondrial dysfunction is suggested to be an early event in Alzheimer's Disease (AD) in which neurons show increased oxidative damage²⁸⁷. Amyloid β precursor protein (A β PP) plaques, which are one of the two hallmarks of AD, accumulates in the mitochondrial protein import channels of human brain, inhibiting the entry of cytochrome oxidase (COX) subunits IV and Vb proteins encoded in the nucleus. Decreased COX activity increases H₂O₂ levels and, in turn, causes oxidative stress^{288,289}. Therefore, discovery of all the proteins playing a role in mitochondrial protein import in mammals might be important for better understanding such diseases related with mitochondrial dysfunction.

Mitochondrial metabolism is reprogrammed during cancer transformation to avoid cell death mechanisms and to utilize the energy metabolism. This reprogramming relies on protein import machineries which were reported to be overexpressed in mitochondria of cancer cells²⁹⁰. A study that reported splice variants of Tim44, which is a component of TIM23 complex, in oncotic thyroid carcinomas suggests that it is likely to have a role in tumorigenesis by impairing the protein import or inducing ROS production²⁹¹. TOM complex proteins were proposed to be interacting with pro- and anti-apoptotic Bcl-2 family proteins. For instance, Tom22 is suggested to be the receptor for pro-apoptotic protein Bax,²⁹² although according to other studies, only the mitochondrial lipid composition might be playing a role in Bax insertion at the MOM²⁹³. Tom20, another TOM subunit is suggested

to have a role in cancer as well. Tom20 interacts with aryl hydrocarbon receptor-interacting protein (AIP)²⁹⁴, which associates with inhibitor of apoptosis protein survivin²⁹⁰.

Genetic selection assays have previously been used for identifying *Saccharomyces cerevisiae* mutants that are unable to perform protein translocation to ER²⁹⁵ or mitochondria²⁹⁶. In this selecting strategy, survival of yeast auxotrophic mutants depends on an organellar targeting signal-containing cytoplasmic enzyme to remain in the cytosol. With this method, they were able to select for yeasts that are unable to perform protein import into ER or mitochondria and identify new mutants^{295,296}. In this work, we aimed to establish a similar selection assay applicable to mammalian cell lines for recovering mutant cells that are unable to operate mitochondrial protein import from a population of mutant cells. To this end, we generated a mammalian cell line that expresses hygromycin B resistance protein hphNT1 containing a mitochondrial targeting signal and localizing to mitochondria. hphNT1 is a phosphotransferase enzyme that detoxifies hygromycin B,²⁹⁷ and we predict it to function only in the cytosol, where hygromycin B is found. Thus, when exposed to hygromycin B, only the mitochondrial import incompetent cells, in which hphNT1 remains in the cytosol, are expected to survive. This selection system will likely enable us to isolate mitochondrial import incompetent cells and to uncover genes expressing novel protein import components, chaperones having role in protein translocation, or genes regulating the mitochondrial protein import in human cells. This approach has a potential to provide a broader perspective for understanding mitochondrial import related diseases or cancer transformation.

5.2 Results

5.2.1 Mammalian cell lines expressing mitochondria or cytoplasm-localized antibiotic resistance protein were generated

Mitochondrial protein import in a healthy mitochondria depends on the presence of a mitochondrial targeting sequence, mostly found on the N- terminus of the precursor protein and a properly functioning translocation machinery²⁵⁷. Relying on this fact, a selection method that is based on an essential protein remaining in the cytosol can be set up in order to select for mitochondrial import mutants. A similar strategy has been previously used to

recover ER²⁹⁵ or mitochondrial²⁹⁶ import mutants in *S. cerevisiae* cells. We used hygromycin B resistance marker protein, hphNT1, targeted to mitochondria to establish a selection system to isolate mitochondrial import mutants upon hygromycin B treatment (Figure 5.1). As a preliminary experiment, we cloned hphNT1 under CMV promoter with a mitochondrial targeting signal (MTS) on its N- terminus along with an enhanced GFP (EGFP)²⁹⁸ at the C-terminus in a mammalian expression vector. We also made a control vector having the same construct, except for the mitochondrial targeting sequence. As expected, when these two plasmids were expressed in human embryonic kidney 293T (HEK293T) cells, EGFP signal was mostly mitochondrial in cells expressing hphNT1 with a mitochondrial targeting signal, while it was diffused to the cytosol in control cells expressing hphNT1 without the signal. However, we could not see a significant difference between the survival of mitochondrial and cytosolic hphNT1 expressing HEK293T cells upon hygromycin B treatment (Figure 5.2), possibly due to varying plasmid copy number among the cell population. In order to control the copy number of hphNT1 sequence and obtain a homogeneous population, we decided to generate cell lines expressing mitochondrial or cytosolic hphNT1 proteins. Upon lentiviral transduction of the same constructs to HEK293T cell lines, we were able to generate two cell lines expressing mitochondria or cytoplasm-localized hphNT1 protein, confirmed by the EGFP localization (Figure 5.3). We named these cells expressing mitochondria or cytoplasm localized hphNT1 protein as MITO-unsorted and CYTO-unsorted respectively. Next, we sorted sub-populations containing low or high EGFP signal, named CYTO-low CYTO-high, MITO-low, and MITO-high (Figure 5.4).

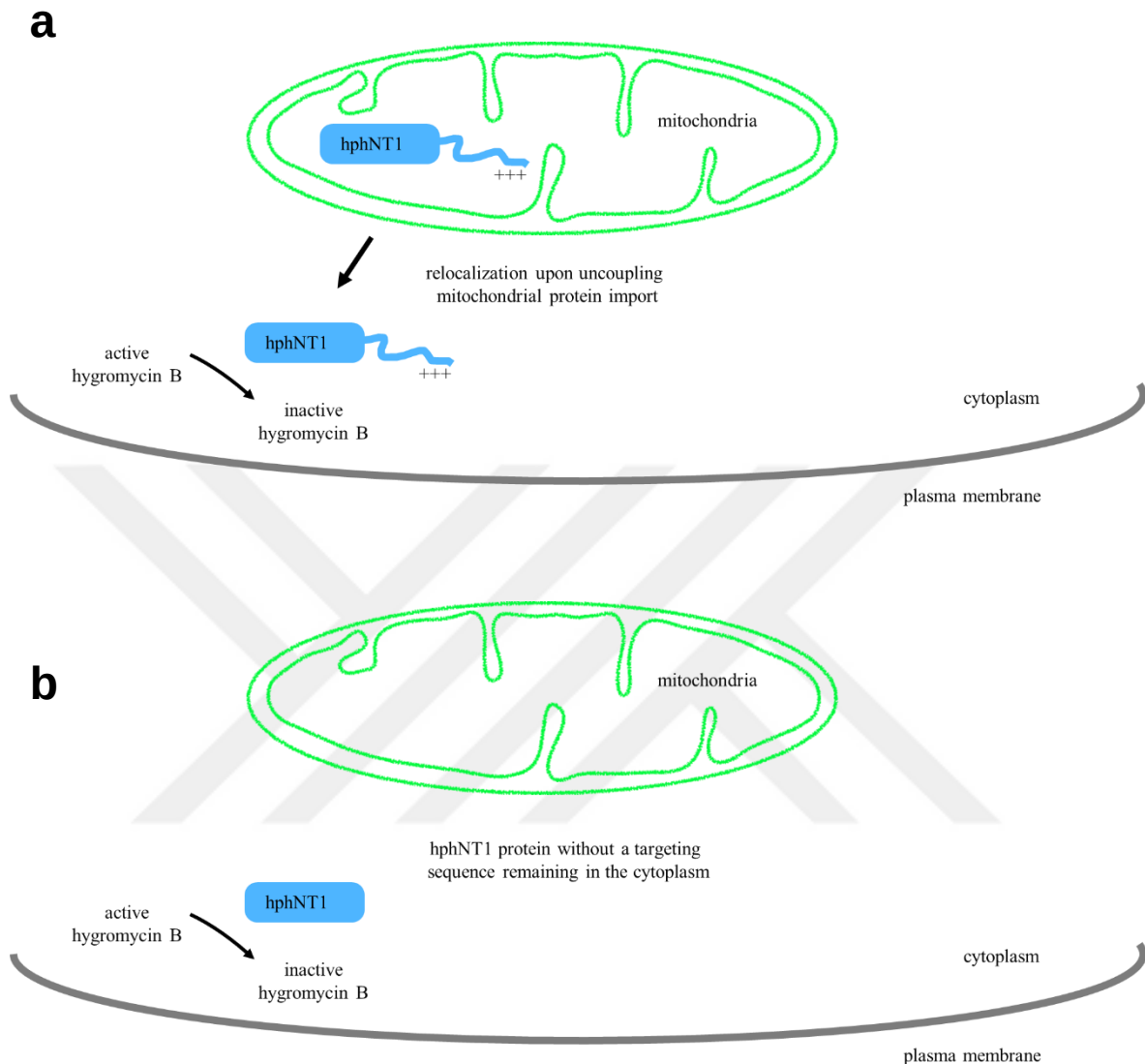


Figure 5.1 A selection strategy based on protein mislocalization may allow recovery of cells in which mitochondrial protein import is blocked. (a) Scheme for selection of cells with a defect in mitochondrial protein import. Upon failure of hphNT1 to be localized to the mitochondria, it may relocate to the cytoplasm and deactivate hygromycin B drug, allowing survival of hygromycin B treated cells. **(b)** In control cells expressing cytoplasm-localized hphNT1, hygromycin B will be inactive, independent of the mitochondrial protein import.

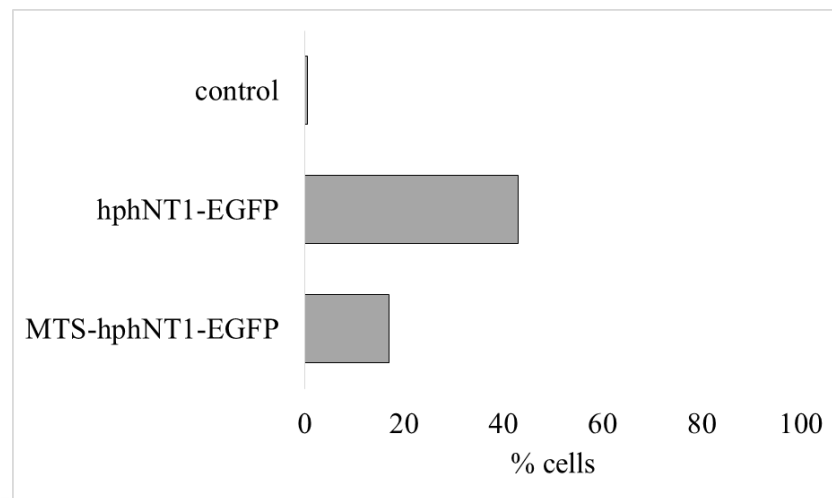


Figure 5.2 Survival of HEK293T cells, which express hphNT1 localized to mitochondria or cytoplasm, in Hygromycin B is not dependent on hphNT1 localization to the cytosol. HEK293T cells were seeded in 12-well plate in complete medium to have 10^5 cells/well concentration and next day transfected with plasmids expressing hphNT1-EGFP (b264), MTS-hphNT1-EGFP (b265), or only treated with transfection reagent (control) with duplicates in each group. Cells were treated with 500 $\mu\text{g/ml}$ hygromycin B for 5 days, while their duplicates remained untreated. At the end of the treatment, cell concentration of hygromycin B treated and untreated wells were counted. The survival percent of each group was calculated by normalizing hygromycin B treated cells according to untreated cells.

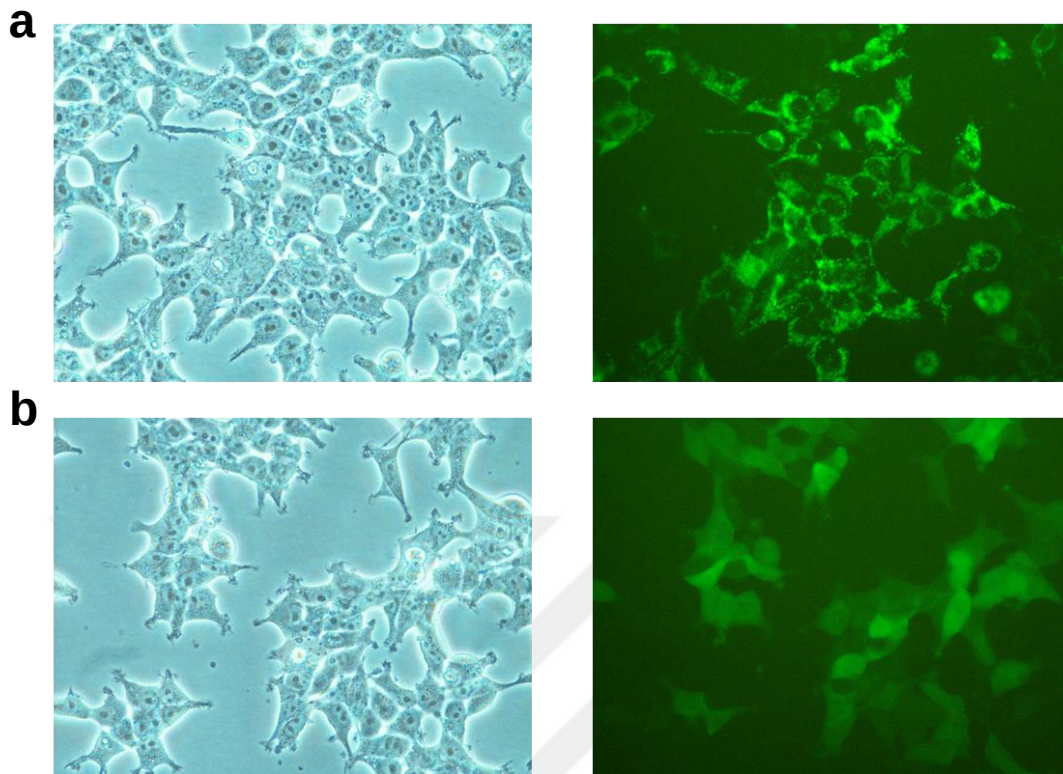


Figure 5.3 HEK293T cells express mitochondria or cytoplasm-localized hphNT1. hphNT1 expression in HEK293T cells after lentivirus-mediated gene transfer. HEK293T cells were infected with lentiviruses containing hphNT1-EGFP fusion protein with (a) and without (b) mitochondrial targeting sequence under CMV promoter. Images were taken using DIC (left) and fluorescence microscopy (right) with 40x magnification.

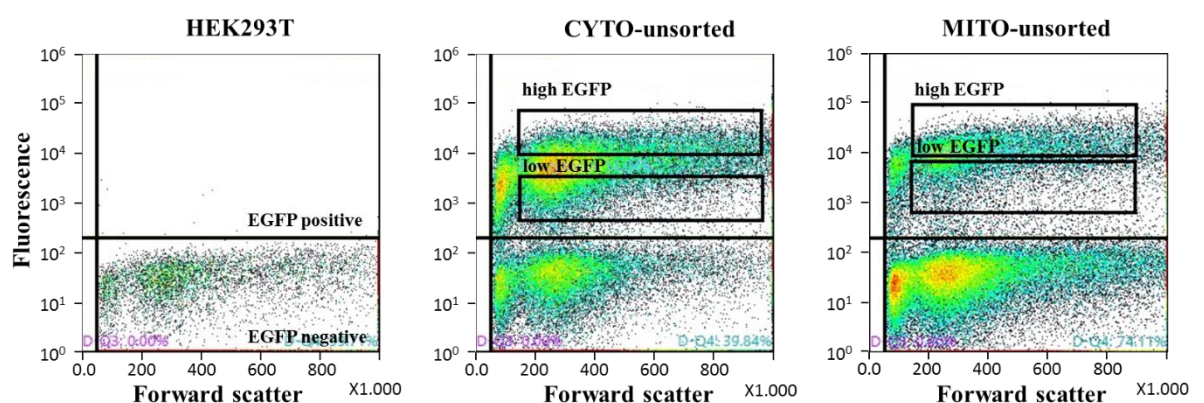


Figure 5.4 CYTO-unsorted and MITO-unsorted cells were sorted into sub-populations with high and low EGFP signal: After the cell populations from Figure 5.3 received 3 passages, they were collected with sorting medium and sorted into 6-well plates using a flow cytometer as sub-populations with high or low EGFP signal. The sorted sub-populations

with high or low EGFP signal are shown with black boxes above which the EGFP level is indicated. WT HEK293T cells are included to show the EGFP negative (top) and positive area (bottom). The EGFP signal was measured using Brilliant Violet 421™ filter.

5.2.2 HEK293T sub-populations expressing mitochondrial or cytosolic hphNT1 are resistant against hygromycin B regardless of the hphNT1 localization

Once we generated the stable cell lines expressing mitochondrial and cytosolic hphNT1, we immediately tested their hygromycin B resistance. In order for the selection method to work, MITO cells should be sensitive to hygromycin B, while relocation of this resistance protein to the cytosol in cells incapable for mitochondrial protein import should render the cells resistant against hygromycin B. Thus, we expected MITO cells to be sensitive and CYTO cells to be resistant against hygromycin B in this test. To investigate the hygromycin B resistance of these two cell populations, we treated their sub-populations having low or high EGFP expression with 500 µg/ml Hygromycin B for 96 hours, followed by 2000 µg/ml hygromycin B treatment for 60 hours. However, MITO-low or MITO-high cells survived even better than CYTO-low or CYTO-high cells, while control wild-type HEK293T cells did not survive at all (Figure 5.5). We concluded that there might be cells with different EGFP expression levels within each sub-populations, resulting in such an unexpected result. To obtain a more homogenous cell population, we continued our experiments with single cell isolates.

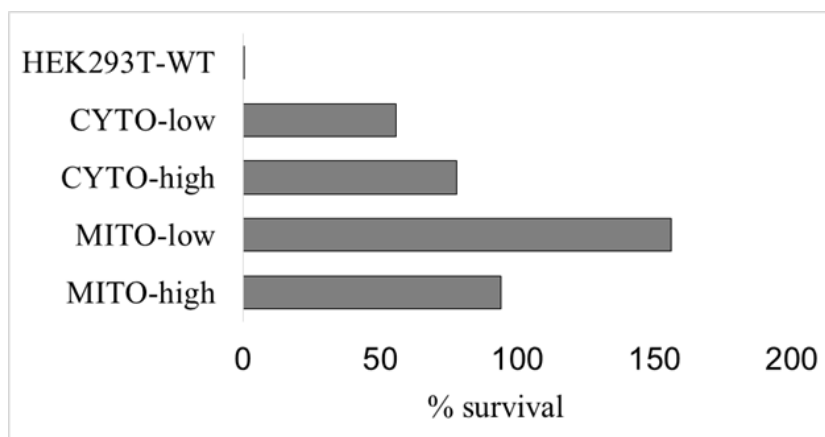


Figure 5.5 Survival of CYTO-low and MITO-low sub-populations in Hygromycin B is not dependent on hphNT1 localization to the cytosol. Individual cell lines were seeded with 243-fold dilution in complete medium from a confluent well to the next with duplicates of equal volume, and cells were treated with 500 µg/ml hygromycin B for 96 hours, followed by 2000 µg/ml hygromycin B treatment for 60 hours, while their duplicates remained untreated. At the end of the treatment, cell concentration in hygromycin B treated and untreated wells were counted. The survival percent of each sub-population was calculated by normalizing hygromycin B treated cells according to untreated cells.

5.2.3 HEK293T single cell isolates expressing mitochondrial or cytosolic hphNT1 might have hphNT1 localization-dependent hygromycin B resistance

Cells may have variable number of DNA copies transferred to their genome upon lentiviral gene transfer²⁹⁹. Having high intracellular hphNT1 concentration might result in a larger cytosolic hphNT1 population even if the protein is mitochondria targeted, and this would interfere with the selection system. To avoid this and control the hphNT1 copy number in the genome, we isolated individual cells with lowest EGFP signal from the previous CYTO-low and MITO-low sub-populations using a cell sorter (Figure 5.6), predicting that the level of EGFP signal would correlate with its genomic copy number. After isolating 192 single cells from mitochondrial and 96 from cytosolic hphNT1 expressing sub-populations and subsequent hygromycin B treatment followed by confirmation of the lentiviral gene transfer by genomic PCR, we selected one cytosolic hphNT1 expressing cell line, named CYTO1, and 6 mitochondrial hphNT1 expressing cell lines named MITO1 to 6 as potential candidates for the selection system. Growth curve analysis of these cell lines upon 1500 µg/ml hygromycin B treatment suggests that they have a hygromycin B sensitivity trend similar to that of wild-type HEK293T cells (Figure 5.7), although we could not detect the EGFP signal of MITO lines with fluorescence microscopy.

Although these results suggest that we might have obtained mitochondrial hphNT1 expressing cell lines that are sensitive to hygromycin B, protein localization with immunostaining, relevant protein expression and accuracy of the genomic sequence remain to be investigated.

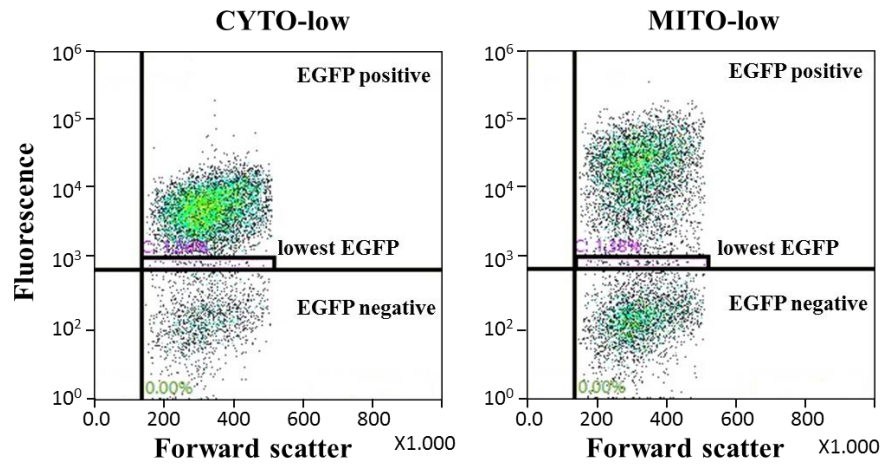


Figure 5.6 CYTO-low and MITO-low cell populations were sorted as single cells with the lowest EGFP signal. Sub-populations from Figure 5.4 were collected with sorting medium and sorted as single cells into 96-well plates using a flow cytometer. The sorted single cells with the lowest EGFP signal are shown with the black box. The EGFP negative (top) and positive area (bottom) are also indicated. The EGFP signal was measured using Brilliant Violet 421™ filter.

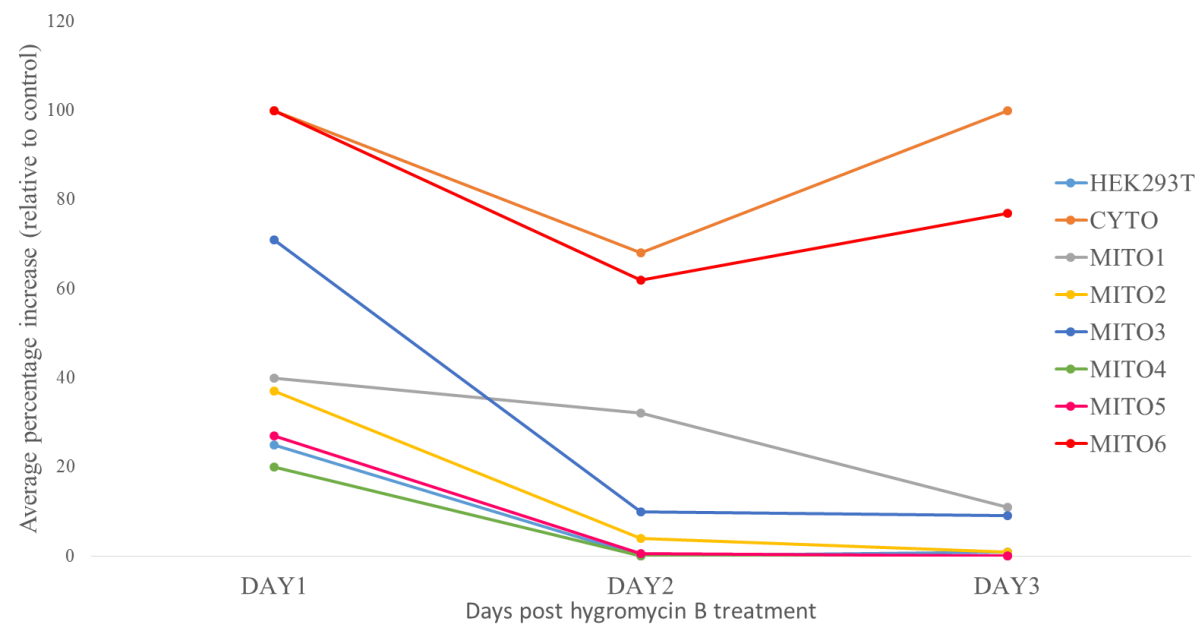


Figure 5.7 Single cell isolates show hpHNT1 localization-dependent proliferation rate upon hygromycin B treatment. Individual cell lines were seeded with 16-fold dilution

in complete medium from a confluent well to the next with duplicates of equal volume, and 24 hours later, on day 0, the cells were treated with 1500 µg/ml hygromycin B containing medium while their duplicates remained untreated. On day 1, 2, and 3 the cell concentration on hygromycin B treated and untreated wells were counted. The proliferation rate of individual cell lines were calculated by normalizing hygromycin B treated cells according to untreated cells.

5.3 Discussion

Our results, which present HEK293T cell lines that show hygromycin B resistance depending on localization of the resistance protein hphNT1, allow us to suggest several candidates for establishing a selection system to isolate mitochondrial protein import mutants, although further confirmations are definitely needed. Accordingly, the cell lines that we have generated have different hygromycin B resistance trends because of the subcellular localization of hphNT1 protein. CYTO cell line that expresses hphNT1 diffused to the cytosol is resistant against hygromycin B antibiotic. On the other hand MITO1 to 6 cell lines expressing mitochondria-localized hphNT1 due to the mitochondrial localization signal found on the N-terminus of the protein are sensitive against hygromycin B to different extents, most of them showing similar trends with wild-type HEK293T cells. Presence of EGFP protein on the N-terminus of either of the hphNT1 variants allowed us to determine the subcellular localization of the protein, while resulting in no interference with the protein function, confirmed by the hygromycin B resistance of CYTO cell line.

As the import rate of folded mitochondria protein precursors are dependent on spontaneous unfolding of the specific protein³⁰⁰, there might be a cytosolic population of mitochondria targeted proteins. Accordingly, the cytosolic population of mitochondria targeted hphNT1 protein might render the cells resistance against hygromycin B. Thus, the intracellular level of hphNT1 protein might be critical for increasing the possibility of this selection system to work. Lentiviral gene transfer methods can integrate variable number of DNA copies in a cell population²⁹⁹. For this reason, isolation of individual cell lines enabled us to control the number of hphNT1 copies in a HEK293T population and to obtain relatively low intracellular hphNT1 concentration so that the mitochondrial import machineries are not

saturated and did not leave a large cytosolic mitochondria-targeted hphNT1 portion. On the other hand, the intracellular localization of hygromycin B is as important as that of hphNT1 while considering the applicability of this selection system.

Hygromycin B is an aminocyclitol antibiotic produced by bacterium *Streptomyces hygroscopicus*³⁰¹ and inhibits protein synthesis by interfering with ribosomal translocation and preventing aminoacyl tRNA synthetase recognition. Given that hygromycin B is also effective in prokaryotes and mitochondrion was once a prokaryotic cell according to the endosymbiotic theory¹⁹⁶, containing prokaryotic lipids on its membranes³⁰², hygromycin B might enter mitochondria. In fact, literature suggests that mitochondria-localized aminoglycosides including hygromycin B can be used as selective antibiotics for mammalian mitochondrial transformation with the help of mitochondria targeted drug resistance proteins, consistent with the potential leakage of the drug into mitochondria³⁰³. hphNT1 is a phosphotransferase enzyme and detoxifies hygromycin B by transferring the phosphate groups²⁹⁷. In other words, if hygromycin B is indeed capable of passing through mitochondrial membranes, then mitochondria-localized hphNT1 can very well detoxify a substantial portion of the intracellular hygromycin B population. Thus, presence of a mitochondrial hphNT1 might render the cells resistance against hygromycin B even if all the intracellular hphNT1 population is found inside the mitochondria.

Overall, our very preliminary results suggest that mitochondrial localization of hphNT1 might prevent mammalian cells from resisting against hygromycin B while its cytosolic localization is protective. However, there are few drawbacks for making this judgement. In order to serve as a good control, CYTO cell line should have comparable hphNT1 expression with MITO cell lines to make sure that the resistance trend that we observed is not quantity but location dependent. Hence, the intracellular CYTO and MITO levels should be compared by means of biochemical analysis. In addition, even if protein levels are comparable, the individual MITO lines might have specific mutations that interfere with protein functionality on the hphNT1 sequence, a potential handicap that cannot be recognized with biochemical assays. Confirmation of the genomic hphNT1 sequence of individual lines is definitely needed to make sure that sensitivity of MITO lines against hygromycin B is not due to a mutation that disrupts hphNT1 functionality. Finally, to confirm location dependent resistance of hphNT1, MITO lines should be tested by mitochondrial import uncoupling drugs or by RNA interference techniques that would transiently halt mitochondrial import

to see whether MITO cells would be resistance against hygromycin B upon relocalization of hphNT1 to the cytosol.

As soon as we confirm the functionality of either of the MITO cell lines for our selection purposes, we will perform genome wide gene knockout on that cell line to potentially target novel mitochondrial translocation components, with the help of genome-wide CRISPR gRNA libraries. Although CRISPR technology has proven efficiency for site-selective, RNA-guided gene knockout by causing double-strand breaks within the target region, it irreversibly deletes the target gene from the genome³⁰⁴. Use of such CRISPR gRNA library might prevent us to detect relevant genes if they are essential for the organism. Detection of such essential genes might be possible by using genome-wide knockdown technologies that would allow us to select cells with lower mitochondrial import competence without killing the organism. CRISPRi technology is the repurposed CRISPR system, in which a catalytically inactive version of Cas9 (dCas9) that lacks endonucleolytic activity. This form of dCas9 can efficiently silence the target gene when co-expressed with a complementary gRNA³⁰⁵. If genome-wide CRISPR knockout does not provide us any novel proteins, possibly due to those proteins being essential for the cell, then we may carry out CRISPRi in order to avoid cell death by deleting any essential component. Finally, the selection of import incompetent cells will be performed to identify potentially new genes related with protein import in mammalian mitochondria.

5.4 Conclusion

These preliminary results presents that we might have generated HEK293T cell lines expressing cytosol (CYTO) or mitochondria-localized hphNT1 (MITO1-6) which renders the cells resistance or sensitive against hygromycin B, respectively, suggesting a hygromycin B resistance trend depending on the cytosolic localization of hphNT1 protein. Although further experiments are needed to confirm this localization dependent resistance, one of these MITO lines might serve as a selection system for isolating mitochondrial import mutant cells upon applying genome-wide knockdown or knockout techniques. Such a selection could potentially allow us to discover novel mammalian mitochondrial import components.

5.5 Materials and Methods

5.5.1 Cell lines and plasmids

A human embryonic kidney cell line, HEK293T (gift from G. İnce-Dunn) was used for this work. Construction details of plasmids and related references of the acquired plasmids are provided in Table 5.1. Oligonucleotides utilized in this work are listed in Table 5.2.

5.5.2 Cell culture conditions and media

HEK293T cells received a passage every 48-96 hours before cell confluency exceeds 90% with 2 to 8-fold dilutions unless indicated otherwise. Cells were grown in complete medium Dulbecco's modified Eagle's medium (DMEM) with 4.5.g/L glucose, 10% (v/v) fetal bovine serum, 2mM L-glutamine and 100U/ml penicillin-streptomycin unless otherwise indicated and incubated at 37 °C in 5% (v/v) CO₂. Antibiotic free medium contains the same ingredients as complete medium except for the penicillin-streptomycin. Sorting medium contains 1% fetal bovine serum in 1x PBS.

5.5.3 Preparation of lentivirus stocks

HEK293T cells were transfected with 2nd generation lentiviral plasmids to generate lentivirus stocks. For production of cytosolic or mitochondrial hphNT1 encoding lentiviral stocks transfer plasmids b320 or b321, viral packaging plasmid gag/pol (gift from G. İnce-Dunn), and envelope plasmid pCMV-VSV-G (gift from G. İnce-Dunn) were used. As a general procedure, one day before transfection, cells were plated on a 25cm² cell culture dishes at 40-50% confluence in antibiotic free medium. After one day's growth when the cells are around 80% confluent, transfections were performed using 1 µg of transfer plasmid, 0.75 µg of viral packaging plasmid, 0.25 µg of envelope plasmid, and 15 µl *TransIT*®-2020 transfection reagent (Mirus) diluted in 600 µl Opti-MEM reduced serum medium (Thermo Fisher). The mixture was incubated at room temperature for 20 minutes before adding to the plates. 48 hours later, when the EGFP signal acquired from the transfer plasmids is visible,

the medium on top was removed, centrifuged, and filtered through 0.2 µm filter to avoid cell remnants. The viral stock was diluted 5-fold and used in viral transduction.

5.5.4 Transfection, transduction and cell sorting

Before transfection, cells were plated on 12-well plates at 40-50% confluence in complete medium. After one day's growth, when the cells are around 80% confluent, transfections were performed using 1 µg of plasmid and 6 µl TransIT®-2020 transfection reagent (Mirus) diluted in 100 µl complete medium for each well. The mixture was incubated at room temperature for 20 minutes before adding to the wells.

Before transduction, cells were plated on a 24-well cell culture dishes at 40-50% confluence. 4 hours later, 500 µl of 5-fold diluted lentiviral solutions were added on wells. 48 hours after the transduction, the cells were relocated into wider dishes to obtain larger cell populations for subsequent experiments.

To analyze or isolate cells containing the EGFP signal, cells suspended in sorting medium were analyzed and a sub-population containing the EGFP signal was sorted into 6-well dish. Single cell isolations were carried out into 96-well plates containing 100 µl complete medium. After sorting into 96-wells, the cells were maintained at 37°C for approximately 2 weeks, changing the media every 48-72 hours and then were relocated into larger dishes to increase cell number. All flow cytometry analysis or cell sorting experiments were performed using Sony SH800 cell sorter.

Table 5.1 Strain used in Chapter 5

Strain	Genotype	Source	Parental Strain	Method Used
BY4743	<i>MATa/α his3Δ1/his3Δ1</i> <i>leu2Δ0/leu2Δ0</i> <i>met15Δ0/MET15</i> <i>LYS2/lys2Δ0</i> <i>ura3Δ0/ura3Δ0</i>	EUROSCARF Y20000		

Table 5.2 Plasmids used in Chapter 5

Plasmid	Name	Source	Construction details
b13	pRS426	231	231
b80	pRS306H	234	234
b167	pEGFP-N1-2 μ -URA3	constructed by Dr. C. Dunn	pEGFP-N1 (gift from G. İnce-Dunn) was amplified using primers 769 and 770. 2 μ -URA3 was amplified from plasmid b13 using primers 771 and 772. PCR products were co-transformed into strain competent <i>S. cerevisiae</i> cells.
b225	OTC	306	Ornithine transcarbamylase (OTC) was a gift from Nicholas Hoogenraad (Addgene plasmid # 71877)
b264	pCMV>OTC(MTS) <i>hphNT1-EGFP</i>	This study	<i>hphNT1</i> ORF was amplified from plasmid b80 using primers 1122 and 1128. PCR products were co-transformed with <i>Bam</i> HI-cut b167 into strain BY4743.
b265	pCMV>OTC(MTS) <i>hphNT1-EGFP</i>	This study	Mitochondrial targeting sequence of OTC was amplified from plasmid b225 using the primers 1124 and 1126. <i>hphNT1</i> ORF was amplified from plasmid b80 using primers 1127 and 1128. PCR products were

Chapter 5: A method to investigate mitochondrial import components in mammalian systems

			co-transformed with <i>Bam</i> HI-cut b167 into strain BY4743.
b299	pLL3.7	307	pLL3.7 was a gift from Luk Parijs (Addgene plasmid # 11795)
b320	pLL3.7-CMV> <i>hphNT1-EGFP</i>	This study	Large fragment (6918 bp) of <i>Nhe</i> I/ <i>Bsr</i> GI-cut plasmid b299 was gel purified and ligated to gel purified small fragment (1787 bp) of <i>Nhe</i> I/ <i>Bsr</i> GI-cut plasmid b264.
b321	pLL3.7-CMV> <i>OTC(MTS)-hphNT1-EGFP</i>	This study	Large fragment (6918 bp) of <i>Nhe</i> I/ <i>Bsr</i> GI-cut plasmid b299 was gel purified and ligated to gel purified small fragment (1853 bp) of <i>Nhe</i> I/ <i>Bsr</i> GI-cut plasmid b265.

Table 5.3 Oligonucleotides used in Chapter 5

Name	Sequence (5'-3')
769	CCTTTTGTGATAATCTCATGACCAAAATCCC
770	ATCTTCACCTAGATCCTTTTAAATTAAAAATG
771	GGGATTTTGGTCATGAGATTATCAAAAAGGTGTATTTAGAAAAATAAACAAATAGGGGTT C
772	CATTTTAAATTTAAAAGGATCTAGGTGAAGATCTGTGCGGTATTTACACACCG
1122	CTCGAGCTCAAGCTTCGAATTCTGCAGTCGATGGGTAAAAAGCCTGAACTCACCGCGACG
1124	CTCGAGCTCAAGCTTCGAATTCTGCAGTCGATGCTGTCTAATTTGAGGATCCTGCTCAAC
1126	CGTCGCGGTGAGTTCAGGCTTTTTACCCATTTTCAGCTGTACTTGACTCTGGACTGGCTT
1127	ATGGGTAAAAAGCCTGAACTCACCGCGACG
1128	GGTGAACAGCTCCTCGCCCTTGCTCACCATTTCTTTGCCCTCGGACGAGTGCTGGGGCG

Chapter 6

IMAGING INTRACELLULAR ENERGY STATUS IN MAMMALIAN CELLS

6.1 Introduction

One important role of mitochondria in a eukaryotic cell is regulating the energy metabolism by cellular respiration. The mitochondrial energy production relies mostly on oxidative phosphorylation, a series of events that produces ATP by transferring electrons to oxygen from compounds with less positive oxidative potentials. The rate of oxidative phosphorylation depends on intracellular ATP and ADP levels. When the cellular energy demand is increased and there is more ADP available in the cell, oxidative respiration rate is also increased to speed up the ATP synthesis ³⁰⁸. Alternatively, in the case of low oxygen levels or mitochondrial dysfunction, the cell meets its energy demands by anaerobic pathways, in which ATP is produced in the cytoplasm ³⁰⁹, a process that generates considerably lower ATP per glucose molecule compared to oxidative respiration. Thus, being responsible for energy production, the mitochondrion is the key determinant of intracellular ATP:ADP ratio, and this ratio is a practical indicative of mitochondrial functionality.

Fluorescent biosensors that can change the excitation wavelength in response to cellular ATP:ADP ratio are commercially available. One of these fluorescent sensors is percevalHR that increases its excitation to 500 nm upon ATP binding and decreases to 420 nm upon ADP binding. The excitation spectrum of the sensor has two distinct peaks at 420 nm and at 500 nm and the fluorescence gives different responds to nucleotide binding. Upon ATP binding, the fluorescence increase for 500 nm excitation while ADP binding increases the fluorescence for 420 nm excitation because of a change in the absorbance of the sensor. The

ratio of the fluorescence at these two excitation wavelengths reports the relative occupancy of PercevalHR by ATP or ADP. This system has previously been confirmed to give functional response to changes in intracellular energy status and allowed cellular imaging by live-cell fluorescence microscopy³¹⁰. For following the energy status of mammalian cells, we introduced this biosensor into the genome of a mammalian cell line. We expect to discriminate between cells containing mitochondria with functional and unhealthy energy metabolism depending on fluorescent signal changes that could be analyzed by means of flow cytometry techniques.

6.2 Results and discussion

A mammalian cell line expressing a fluorescent-biosensor for detecting intracellular ATP:ADP ratio was generated

To take advantage of this fluorescent biosensor for following intracellular energy status, we introduced the relevant gene encoding the biosensor into HEK293T cells with lentiviral transduction and confirmed the presence of fluorescent protein by fluorescence microscopy. Next, we isolated single cells with cell sorter, and continued with one cell line confirmed to have the fluorescent signal and named PRCV (Figure 6.1). Finally we tested the capacity of the fluorescent biosensor to decrease or increase its excitation upon binding to ADP or ATP. We treated PRCV cell line with 1500, 1000, 750, or 500 ng/ml tetracycline for 2 days to block oxidative phosphorylation. The treatments were carried out in complete medium with galactose instead of glucose so that intracellular ATP levels would decrease as glycolytic metabolism of galactose produces no net ATP, while that of one glucose molecule yields 2 net ATP³¹¹. Next, we analyzed fluorescent levels in these populations on flow cytometer. However, we could not observe a difference in fluorescent signals compared to control population (Figure 6.2). Yet, response of the PRCV cell line towards other respiration uncouplers such as oligomycin³¹² remains to be tested.

Once we confirm that percevalHR response against changes in intracellular energy status is significant enough to be detected and discriminated by a cell sorter, we can use this system to detect cells with mitochondrial dysfunction. Furthermore, by means of genome-wide

knockdown or knockout methods, PRCV cell line can be used to discriminate cells that have respiration defects and detect previously undiscovered genes having role in eukaryotic energy metabolism. Overall, this systems might allow us to examine metabolism of mammalian mitochondria in greater detail.

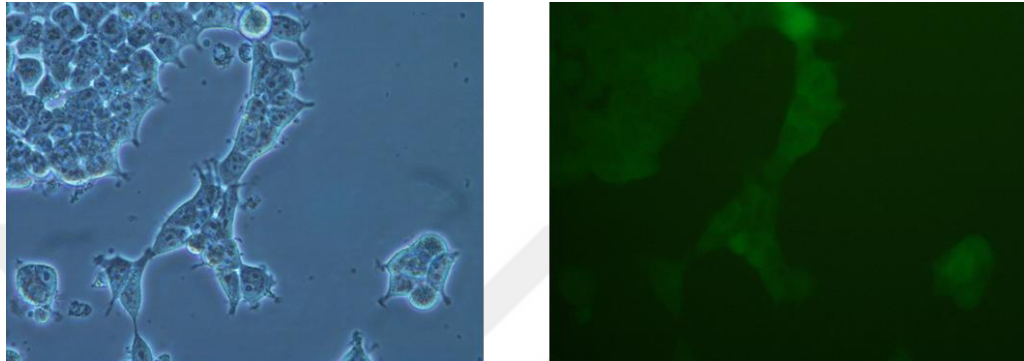


Figure 6.1 PRCV cells express fluorescent-biosensor perceiverHR. perceiverHR expression in PRCV cells after lentivirus-mediated gene transfer and subsequent single cell isolation. PRCV cells were imaged using DIC (left) and fluorescence microscopy (right) with 40x magnification.

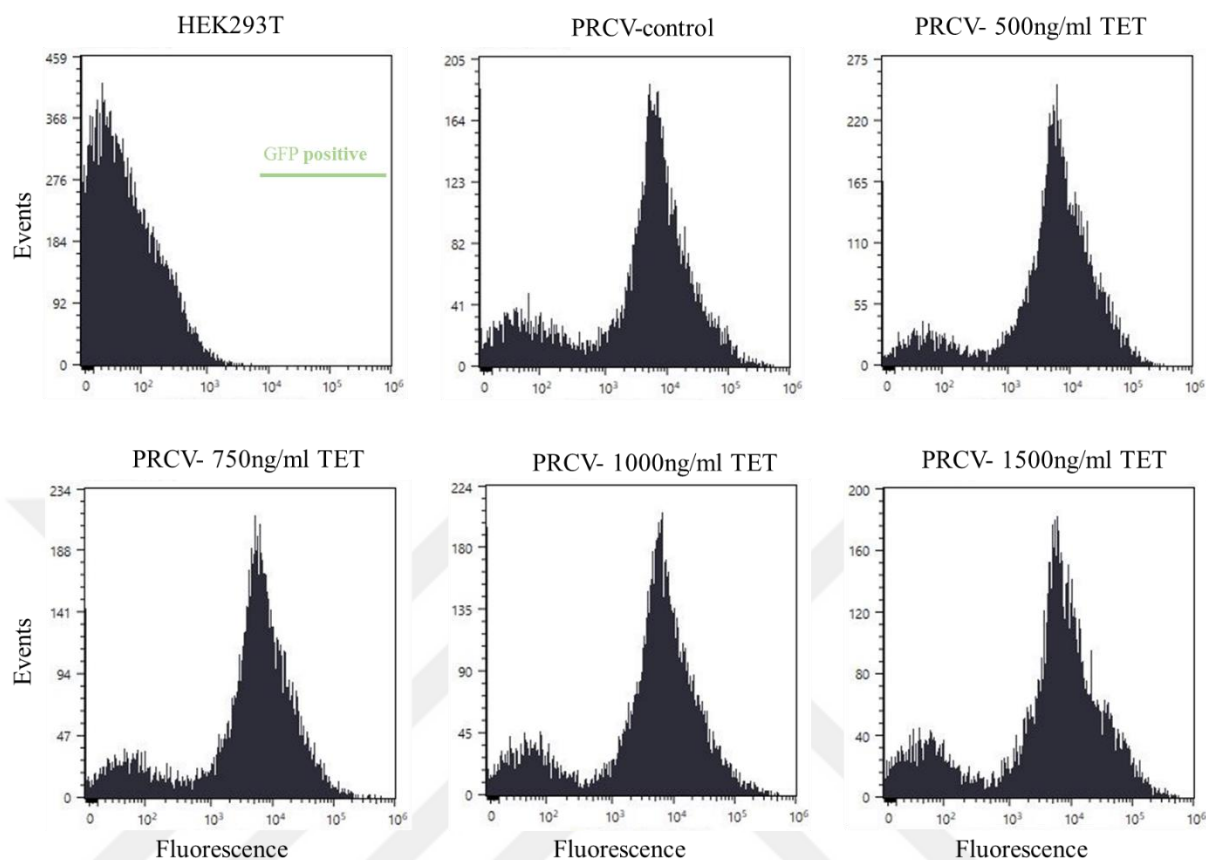


Figure 6.2 PRCV cells do not change fluorescent signal upon tetracycline treatment.

PRCV cells were seeded in 12-well plate in galactose medium and 24 hours later, treated with indicated amount of tetracycline (TET) or without tetracycline as control for 2 days. WT HEK293T cells also grown in galactose medium were included to show the GFP positive area. At the end of day 2, cells were collected and the GFP signal was measured with flow cytometer, using FITC filter.

6.3 Materials and Methods

6.3.1 Cell lines and plasmids

A human embryonic kidney cell line, HEK293T (gift from G. İnce-Dunn) was used for this work. Construction details of plasmids and related references of the acquired plasmids are provided in Table 6.1.

6.3.2 Cell culture conditions and media

HEK293T cells received a passage every 48-96 hours before cell confluency exceeds 90% with 2 to 8-fold dilutions unless indicated otherwise. Cells were grown in complete medium Dulbecco's modified Eagle's medium (DMEM) with 4.5.g/L glucose, 10% (v/v) fetal bovine serum, 2mM L-glutamine and 100U/ml penicillin-streptomycin unless otherwise indicated and incubated at 37 °C in 5% (v/v) CO₂. Complete medium with galactose contains the same ingredients as complete medium with glucose except it contains 5mM galactose instead of glucose. Antibiotic free medium contains the same ingredients as complete medium except for the penicillin-streptomycin. Sorting medium contains 1% fetal bovine serum in 1x PBS.

6.3.3 Preparation of lentivirus stocks

HEK293T cells were transfected with 2nd generation lentiviral plasmids to generate lentivirus stocks. For percevalHR encoding lentivirus production, stocks transfer plasmid b271, viral packaging plasmid b356, and envelope plasmid b324 were used. As a general procedure, one day before transfection, cells were plated on a 25cm² cell culture dishes at 40-50% confluence in antibiotic free medium. After one day's growth when the cells are around 80% confluent, transfections were performed using 1 µg of transfer plasmid, 0.75 µg of viral packaging plasmid, 0.25 µg of envelope plasmid, and 15 µl *TransIT*®-2020 transfection reagent (Mirus) diluted in 600 µl Opti-MEM reduced serum medium (Thermo Fisher). The mixture was incubated at room temperature for 20 minutes before adding to the plates. 48 hours later when the EGFP signal acquired from the transfer plasmids is visible, the medium on top was removed, centrifuged, and filtered through 0.2 µm filter to avoid cell remnants. The obtained viral stock was used in viral transduction.

6.3.4 Transduction and cell sorting

Before transduction, cells were plated on a 24-well cell culture dishes at 40-50% confluence. 4 hours later, 500 µl of 5-fold diluted lentiviral solutions were added on wells.

48 hours after the transduction, the cells were relocated into wider dishes to obtain larger cell populations for subsequent experiments.

Analysis of the EGFP signal in PRCV cells were made using a flow cytometer with Brilliant Violet 421™ filter, upon suspension in sorting medium. Single cell isolations were carried out into 96-well plates containing 100 µl complete medium. After sorting into 96-wells, the cells were maintained at 37°C for approximately 2 weeks, changing the media every 48-72 hours and then were relocated into larger dishes to increase cell number. All flow cytometry analysis or cell sorting experiments were performed using Sony SH800 cell sorter.

Table 6.1 Plasmids used in Chapter 6

Plasmid	Name	Source	Construction details
b271	FUGW-PercevalHR	310	FUGW-PercevalHR was a gift from Gary Yellen (Addgene plasmid # 49083)
b324	pMD2.G		pMD2.G was a gift from Didier Trono (Addgene plasmid # 12259)
b356	psPAX2		psPAX2 was a gift from Didier Trono (Addgene plasmid # 12260)

BIBLIOGRAPHY

1. Dudek, J., Rehling, P. & van der Laan, M. Mitochondrial protein import: Common principles and physiological networks. *Biochim. Biophys. Acta - Mol. Cell Res.* **1833**, 274–285 (2013).
2. Kühlbrandt, W. Structure and function of mitochondrial membrane protein complexes. *BMC Biol.* **13**, 89 (2015).
3. Schmidt, O., Pfanner, N. & Meisinger, C. Mitochondrial protein import: from proteomics to functional mechanisms. *Nat. Rev. Mol. Cell Biol.* **11**, 655–667 (2010).
4. Gray, M. W. Mosaic nature of the mitochondrial proteome: Implications for the origin and evolution of mitochondria. *Proc. Natl. Acad. Sci.* **112**, 10133–10138 (2015).
5. Gabaldón, T. & Huynen, M. A. From endosymbiont to host-controlled organelle: The hijacking of mitochondrial protein synthesis and metabolism. *PLoS Comput. Biol.* **3**, 2209–2218 (2007).
6. Gray, M. W. Mitochondrial evolution. *Cold Spring Harb. Perspect. Biol.* **4**, (2012).
7. de Duve, C. The origin of eukaryotes: a reappraisal. *Nat. Rev. Genet.* **8**, 395–403 (2007).
8. Kurland, C. G. Genomics and the Irreducible Nature of Eukaryote Cells. *Science (80-.).* **312**, 1011–1014 (2006).
9. Rapaport, D. & Neupert, W. Biogenesis of Tom40, core component of the TOM complex of mitochondria. *J. Cell Biol.* **146**, 321–331 (1999).
10. Model, K. *et al.* Multistep assembly of the protein import channel of the mitochondrial outer membrane. *Nat. Struct. Biol.* **8**, 361–370 (2001).
11. Chacinska, A., Koehler, C. M., Milenkovic, D., Lithgow, T. & Pfanner, N. Importing Mitochondrial Proteins: Machineries and Mechanisms. *Cell* **138**, 628–644 (2009).

Bibliography

12. Margulis, L. Symbiotic theory of the origin of eukaryotic organelles; criteria for proof. *Symp. Soc. Exp. Biol.* **29**, 21–38 (1975).
13. Sagan, L. On the origin of mitosing cells. *J. Theor. Biol.* **14**, 225–IN6 (1967).
14. Baum, D. A. & Baum, B. An inside-out origin for the eukaryotic cell. *BMC Biol.* **12**, 76 (2014).
15. Margulis, L., Chapman, M., Guerrero, R. & Hall, J. The last eukaryotic common ancestor (LECA): acquisition of cytoskeletal motility from aerotolerant spirochetes in the Proterozoic Eon. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 13080–13085 (2006).
16. Martin, W. F., Garg, S. & Zimorski, V. Endosymbiotic theories for eukaryote origin. *Philos. Trans. R. Soc. B Biol. Sci.* **370**, 20140330 (2015).
17. Cavalier-Smith, T. The number of symbiotic origins of organelles. *BioSystems* **28**, 91–106 (1992).
18. Slominski, P. P. & Ephrussi, B. Subcellular units involved in the synthesis of respiratory enzymes in yeast. *Nature* **176**, 1207–8 (1955).
19. Chen, S. Y., Ephrussi, B. & Hottinguer, H. Nature genetique des mutants a deficiencie respiratoire de la souche B-II de la levure de boulangerie. *Heredity (Edinb)*. **4**, 337–351 (1950).
20. NASS, M. M. & NASS, S. INTRAMITOCHONDRIAL FIBERS WITH DNA CHARACTERISTICS. I. FIXATION AND. *J. Cell Biol.* **19**, 593–611 (1963).
21. Schatz, G., Haslbrunner, E. & Tuppy, H. Deoxyribonucleic acid associated with yeast mitochondria. *Biochem. Biophys. Res. Commun.* **15**, 127–132 (1964).
22. Wintersberger, E. & Tuppy, H. Mitochondria synthesize RNA. *Biochem Zeitschrift* **341**, 399–408 (1965).
23. Gray, M. W. The Bacterial Ancestry of Plastids and Mitochondria. *Bioscience* **33**, 693–699 (1983).
24. Anderson, S. *et al.* Sequence and organization of the human mitochondrial genome. *Nature* **290**, 457–465 (1981).
25. Gray, M. W. *et al.* Genome structure and gene content in protist mitochondrial

- DNAs. *Nucleic Acids Res.* **26**, 865–878 (1998).
26. Feagin, J. E. Mitochondrial genome diversity in parasites. *International Journal for Parasitology* **30**, 371–390 (2000).
27. Sloan, D. B. *et al.* Rapid evolution of enormous, multichromosomal genomes in flowering plant mitochondria with exceptionally high mutation rates. *PLoS Biol.* **10**, (2012).
28. Kurland, C. G. & Andersson, S. G. E. Origin and Evolution of the Mitochondrial Proteome. *Microbiol. Mol. Biol. Rev.* **64**, 786–820 (2000).
29. Schmidt, O., Pfanner, N. & Meisinger, C. Mitochondrial protein import: from proteomics to functional mechanisms. *Nat. Rev. Mol. Cell Biol.* **11**, 655–667 (2010).
30. Richly, E., Chinnery, P. F. & Leister, D. Evolutionary diversification of mitochondrial proteomes: Implications for human disease. *Trends in Genetics* **19**, 356–362 (2003).
31. Adams, K. L. & Palmer, J. D. Evolution of mitochondrial gene content: Gene loss and transfer to the nucleus. *Mol. Phylogenet. Evol.* **29**, 380–395 (2003).
32. Hewitt, V., Alcock, F. & Lithgow, T. Minor modifications and major adaptations: The evolution of molecular machines driving mitochondrial protein import. *Biochimica et Biophysica Acta - Biomembranes* **1808**, 947–954 (2011).
33. Dolezal, P., Likic, V., Tachezy, J. & Lithgow, T. Evolution of the molecular machines for protein import into mitochondria. *Science* **313**, 314–8 (2006).
34. Vögtle, F. N. *et al.* Global Analysis of the Mitochondrial N-Proteome Identifies a Processing Peptidase Critical for Protein Stability. *Cell* **139**, 428–439 (2009).
35. Diekert, K., Kispal, G., Guiard, B. & Lill, R. An internal targeting signal directing proteins into the mitochondrial intermembrane space. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 11752–11757 (1999).
36. Borgese, N., Gazzoni, I., Barberi, M., Colombo, S. & Pedrazzini, E. Targeting of a tail-anchored protein to endoplasmic reticulum and mitochondrial outer membrane by independent but competing pathways. *Mol Biol Cell* **12**, 2482–2496 (2001).
37. Dudek, J., Rehling, P. & van der Laan, M. Mitochondrial protein import: Common

- principles and physiological networks. *Biochim. Biophys. Acta - Mol. Cell Res.* **1833**, 274–285 (2013).
38. Dekker, P. J. *et al.* Preprotein translocase of the outer mitochondrial membrane: molecular dissection and assembly of the general import pore complex. *Mol. Cell. Biol.* **18**, 6515–6524 (1998).
 39. Hill, K. *et al.* Tom40 forms the hydrophilic channel of the mitochondrial import pore for preproteins. *Nature* **395**, 516–521 (1998).
 40. Renthall, R. Helix insertion into bilayers and the evolution of membrane proteins. *Cellular and Molecular Life Sciences* **67**, 1077–1088 (2010).
 41. Kol, S., Nouwen, N. & Driessen, A. J. M. Mechanisms of YidC-mediated insertion and assembly of multimeric membrane protein complexes. *Journal of Biological Chemistry* **283**, 31269–31273 (2008).
 42. Urbanus, M. L. *et al.* Targeting, insertion, and localization of Escherichia coli YidC. *J. Biol. Chem.* **277**, 12718–12723 (2002).
 43. Hell, K., Herrmann, J. M., Pratje, E., Neupert, W. & Stuart, R. A. Oxa1p, an essential component of the N-tail protein export machinery in mitochondria. *Proc. Natl. Acad. Sci.* **95**, 2250–2255 (1998).
 44. Veenendaal, A. K. J., Van Der Does, C. & Driessen, A. J. M. The protein-conducting channel SecYEG. *Biochimica et Biophysica Acta - Molecular Cell Research* **1694**, 81–95 (2004).
 45. Mokranjac, D. & Neupert, W. Thirty years of protein translocation into mitochondria: Unexpectedly complex and still puzzling. *Biochimica et Biophysica Acta - Molecular Cell Research* **1793**, 33–41 (2009).
 46. Scotto, A. W. & Zakim, D. Reconstitution of membrane proteins. Spontaneous incorporation of integral membrane proteins into preformed bilayers of pure phospholipid. *J. Biol. Chem.* **263**, 18500–18506 (1988).
 47. Rabu, C., Wipf, P., Brodsky, J. L. & High, S. A precursor-specific role for Hsp40/Hsc70 during tail-anchored protein integration at the endoplasmic reticulum. *J. Biol. Chem.* **283**, 27504–27513 (2008).

Bibliography

48. Krumpe, K. *et al.* Ergosterol content specifies targeting of tail-anchored proteins to mitochondrial outer membranes. *Mol. Biol. Cell* **23**, 3927–3935 (2012).
49. Brambillasca, S., Yabal, M., Makarow, M. & Borgese, N. Unassisted translocation of large polypeptide domains across phospholipid bilayers. *J. Cell Biol.* **175**, 767–777 (2006).
50. He, K., Ludtke, S. J., Heller, W. T. & Huang, H. W. Mechanism of alamethicin insertion into lipid bilayers. *Biophys. J.* **71**, 2669–79 (1996).
51. Lin, J. & Baumgaertner, A. Stability of a melittin pore in a lipid bilayer: a molecular dynamics study. *Biophys. J.* **78**, 1714–1724 (2000).
52. Pino-Angeles, A., Leveritt, J. M. & Lazaridis, T. Pore Structure and Synergy in Antimicrobial Peptides of the Magainin Family. *PLoS Comput. Biol.* **12**, (2016).
53. Gallucci, E., Meleleo, D., Micelli, S. & Picciarelli, V. Magainin 2 channel formation in planar lipid membranes: the role of lipid polar groups and ergosterol. *Eur. Biophys. J.* **32**, 22–32 (2003).
54. Haugen, H. S., Kristiansen, P. E., Fimland, G. & Nissen-Meyer, J. Mutational analysis of the class IIa bacteriocin curvacin A and its orientation in target cell membranes. *Appl. Environ. Microbiol.* **74**, 6766–6773 (2008).
55. Cascales, E. *et al.* Colicin Biology. *Microbiol. Mol. Biol. Rev.* **71**, 158–229 (2007).
56. Borgese, N., Colombo, S. & Pedrazzini, E. The tale of tail-anchored proteins: Coming from the cytosol and looking for a membrane. *Journal of Cell Biology* **161**, 1013–1019 (2003).
57. Nyathi, Y., Wilkinson, B. M. & Pool, M. R. Co-translational targeting and translocation of proteins to the endoplasmic reticulum. *Biochimica et Biophysica Acta - Molecular Cell Research* **1833**, 2392–2402 (2013).
58. Linstedt, a D. & Hauri, H. P. Giantin, a novel conserved Golgi membrane protein containing a cytoplasmic domain of at least 350 kDa. *Mol. Biol. Cell* **4**, 679–693 (1993).
59. Jääntti, J. *et al.* Membrane insertion and intracellular transport of yeast syntaxin Sso2p in mammalian cells. *J. Cell Sci.* **107** (Pt 1, 3623–33 (1994).

60. Motley, A. M. & Hettema, E. H. Yeast peroxisomes multiply by growth and division. *J. Cell Biol.* **178**, 399–410 (2007).
61. Dimitrov, L., Lam, S. K. & Schekman, R. The role of the endoplasmic reticulum in peroxisome biogenesis. *Cold Spring Harbor perspectives in biology* **5**, (2013).
62. van der Zand, A., Braakman, I. & Tabak, H. F. Peroxisomal membrane proteins insert into the endoplasmic reticulum. *Mol. Biol. Cell* **21**, 2057–65 (2010).
63. Borgese, N. & Fasana, E. Targeting pathways of C-tail-anchored proteins. *Biochimica et Biophysica Acta - Biomembranes* **1808**, 937–946 (2011).
64. Plath, K., Mothes, W., Wilkinson, B. M., Stirling, C. J. & Rapoport, T. A. Signal sequence recognition in posttranslational protein transport across the yeast ER membrane. *Cell* **94**, 795–807 (1998).
65. Kutay, U., Ahnert-Hilger, G., Hartmann, E., Wiedenmann, B. & Rapoport, T. A. Transport route for synaptobrevin via a novel pathway of insertion into the endoplasmic reticulum membrane. *EMBO J.* **14**, 217–23 (1995).
66. Steel, G. J., Brownsword, J. & Stirling, C. J. Tail-anchored protein insertion into yeast ER requires a novel posttranslational mechanism which is independent of the SEC machinery. *Biochemistry* **41**, 11914–11920 (2002).
67. Abell, B. M. *et al.* Tail-anchored and signal-anchored proteins utilize overlapping pathways during membrane insertion. *J. Biol. Chem.* **278**, 5669–5678 (2003).
68. Aviram, N. *et al.* The SND proteins constitute an alternative targeting route to the endoplasmic reticulum. *Nature* **540**, 134–138 (2016).
69. Stefanovic, S. & Hegde, R. S. Identification of a Targeting Factor for Posttranslational Membrane Protein Insertion into the ER. *Cell* **128**, 1147–1159 (2007).
70. Schuldiner, M. *et al.* Exploration of the function and organization of the yeast early secretory pathway through an epistatic miniarray profile. *Cell* **123**, 507–519 (2005).
71. Schuldiner, M. *et al.* The GET Complex Mediates Insertion of Tail-Anchored Proteins into the ER Membrane. *Cell* **134**, 634–645 (2008).
72. Rao, M. *et al.* Multiple selection filters ensure accurate tail-anchored membrane

- protein targeting. *Elife* **5**, (2016).
73. Enoch, H. G., Fleming, P. J. & Strittmatter, P. The binding of cytochrome b5 to phospholipid vesicles and biological membranes. Effect of orientation on intermembrane transfer and digestion by carboxypeptidase Y. *J Biol Chem* (1979).
 74. Brambillasca, S. *et al.* Transmembrane topogenesis of a tail-anchored protein is modulated by membrane lipid composition. *EMBO J.* **24**, 2533–2542 (2005).
 75. Colombo, S. F., Longhi, R. & Borgese, N. The role of cytosolic proteins in the insertion of tail-anchored proteins into phospholipid bilayers. *J. Cell Sci.* **122**, 2383–92 (2009).
 76. Kornmann, B. & Walter, P. ERMES-mediated ER-mitochondria contacts: molecular hubs for the regulation of mitochondrial biology. *J. Cell Sci.* **123**, 1389–1393 (2010).
 77. Ji, W. *et al.* Receptor-mediated Drp1 oligomerization on endoplasmic reticulum. *J. Cell Biol.* **216**, 4123–4139 (2017).
 78. Youle, R. J. & Strasser, A. The BCL-2 protein family: opposing activities that mediate cell death. *Nat. Rev. Mol. Cell Biol.* **9**, 47–59 (2008).
 79. Brahmabhatt, H., Uehling, D., Al-awar, R., Leber, B. & Andrews, D. Small molecules reveal an alternative mechanism of Bax activation. *Biochem. J.* **0**, 1073–1083 (2016).
 80. Setoguchi, K., Otera, H. & Mihara, K. Cytosolic factor- and TOM-independent import of C-tail-anchored mitochondrial outer membrane proteins. *EMBO J.* **25**, 5635–47 (2006).
 81. Kaufmann, T. *et al.* Characterization of the signal that directs Bcl-xL, but not Bcl-2, to the mitochondrial outer membrane. *J. Cell Biol.* **160**, 53–64 (2003).
 82. Maity, A., Sinha, S., Ganguly, D. & Ghosh Dastidar, S. C-terminal tail insertion of Bcl-xL in membrane occurs via partial unfolding and refolding cycle associating microsolvation. *Phys Chem Chem Phys* **18**, 24095–24105 (2016).
 83. Koch, A., Yoon, Y., Bonekamp, N. A., McNiven, M. A. & Schrader, M. A role for Fis1 in both mitochondrial and peroxisomal fission in mammalian cells. *Mol. Biol.*

- Cell* **16**, 5077–5086 (2005).
84. Elgersma, Y. *et al.* Overexpression of Pex15p, a phosphorylated peroxisomal integral membrane protein required for peroxisome assembly in *S. cerevisiae*, causes proliferation of the endoplasmic reticulum membrane. *EMBO J.* **16**, 7326–7341 (1997).
 85. Kemper, C. *et al.* Integration of tail-anchored proteins into the mitochondrial outer membrane does not require any known import components. *J. Cell Sci.* **121**, 1990–1998 (2008).
 86. Yagita, Y., Hiromasa, T. & Fujiki, Y. Tail-anchored PEX26 targets peroxisomes via a PEX19-dependent and TRC40-independent class I pathway. *J. Cell Biol.* **200**, 651–666 (2013).
 87. Henderson, M. P. A., Hwang, Y. T., Dyer, J. M., Mullen, R. T. & Andrews, D. W. The C-terminus of cytochrome *b*₅ confers endoplasmic reticulum specificity by preventing spontaneous insertion into membranes. *Biochem. J.* **401**, 701–709 (2007).
 88. Kuroda, R. *et al.* Charged amine acids at the carboxyl-terminal portions determine the intracellular locations of two isoforms of cytochrome *b*₅. *J. Biol. Chem.* **273**, 31097–31102 (1998).
 89. Isenmann, S., Khew-Goodall, Y., Gamble, J., Vadas, M. & Wattenberg, B. W. A splice-isoform of vesicle-associated membrane protein-1 (VAMP-1) contains a mitochondrial targeting signal. *Mol. Biol. Cell* **9**, 1649–1660 (1998).
 90. Whitley, P., Grahn, E., Kutay, U., Rapoport, T. A. & Von Heijne, G. A 12-residue-long polyleucine tail is sufficient to anchor synaptobrevin to the endoplasmic reticulum membrane. *J. Biol. Chem.* **271**, 7583–7586 (1996).
 91. Yang, M., Ellenberg, J., Bonifacino, J. S. & Weissman, A. M. The transmembrane domain of a carboxyl-terminal anchored protein determines localization to the endoplasmic reticulum. *J. Biol. Chem.* **272**, 1970–1976 (1997).
 92. Keskin, A., Akdogan, E. & Dunn, C. D. Deep mutational scanning reveals tail anchor characteristics important for mitochondrial targeting. (2016).

doi:10.1101/045351

93. Buentzel, J., Vilardi, F., Lotz-Havla, A., Gärtner, J. & Thoms, S. Conserved targeting information in mammalian and fungal peroxisomal tail-anchored proteins. *Sci. Rep.* **5**, 17420 (2015).
94. Keskin, A., Akdoğan, E. & Dunn, C. D. Evidence for amino acid snorkeling from a high-resolution, in vivo analysis of Fis1 tail-anchor insertion at the mitochondrial outer membrane. *Genetics* **205**, 691–705 (2017).
95. Stojanovski, D., Koutsopoulos, O. S., Okamoto, K. & Ryan, M. T. Levels of human Fis1 at the mitochondrial outer membrane regulate mitochondrial morphology. *J. Cell Sci.* **117**, 1201–1210 (2004).
96. Cymer, F., Von Heijne, G. & White, S. H. Mechanisms of integral membrane protein insertion and folding. *Journal of Molecular Biology* **427**, 999–1022 (2015).
97. Gavel, Y., Nilsson, L. & von Heijne, G. Mitochondrial targeting sequences why ‘non-amphiphilic’ peptides may still be amphiphilic. *FEBS Lett.* **235**, 173–177 (1988).
98. Mozdy, A. D., McCaffery, J. M. & Shaw, J. M. Dnm1p GTPase-mediated mitochondrial fission is a multi-step process requiring the novel integral membrane component Fis1p. *J. Cell Biol.* **151**, 367–379 (2000).
99. James, D. I., Parone, P. A., Mattenberger, Y. & Martinou, J. C. hFis1, a novel component of the mammalian mitochondrial fission machinery. *J. Biol. Chem.* **278**, 36373–36379 (2003).
100. Otera, H. *et al.* Mff is an essential factor for mitochondrial recruitment of Drp1 during mitochondrial fission in mammalian cells. *J. Cell Biol.* **191**, 1141–1158 (2010).
101. Loson, O. C., Song, Z., Chen, H. & Chan, D. C. Fis1, Mff, MiD49, and MiD51 mediate Drp1 recruitment in mitochondrial fission. *Mol. Biol. Cell* **24**, 659–667 (2013).
102. Shen, Q. *et al.* Mutations in Fis1 disrupt orderly disposal of defective mitochondria. *Mol. Biol. Cell* **25**, 145–159 (2013).

Bibliography

103. Cervený, K. L., Tamura, Y., Zhang, Z., Jensen, R. E. & Sesaki, H. Regulation of mitochondrial fusion and division. *Trends in Cell Biology* **17**, 563–569 (2007).
104. Motley, A. M., Ward, G. P. & Hettema, E. H. Dnm1p-dependent peroxisome fission requires Caf4p, Mdv1p and Fis1p. *J. Cell Sci.* **121**, 1633–40 (2008).
105. Kornmann, B. *et al.* An ER-Mitochondria Tethering Complex Revealed by a Synthetic Biology Screen. *Science* (80-.). **325**, 477–481 (2009).
106. Keskin, A. A genetic approach to reveal determinants of mitochondrial tail-anchored protein targeting. (Koç University, 2016).
107. Kuravi, K. Dynamin-related proteins Vps1p and Dnm1p control peroxisome abundance in *Saccharomyces cerevisiae*. *J. Cell Sci.* **119**, 3994–4001 (2006).
108. Van Der Zand, A., Gent, J., Braakman, I. & Tabak, H. F. Biochemically distinct vesicles from the endoplasmic reticulum fuse to form peroxisomes. *Cell* **149**, 397–409 (2012).
109. Liu, Y., Yagita, Y. & Fujiki, Y. Assembly of Peroxisomal Membrane Proteins via the Direct Pex19p-Pex3p Pathway. *Traffic* **17**, 433–455 (2016).
110. Kim, P. K. & Hettema, E. H. Multiple pathways for protein transport to peroxisomes. *Journal of Molecular Biology* **427**, 1176–1190 (2015).
111. Agrawal, G. & Subramani, S. De novo peroxisome biogenesis: Evolving concepts and conundrums. *Biochimica et Biophysica Acta - Molecular Cell Research* **1863**, 892–901 (2016).
112. Grimm, I., Erdmann, R. & Girzalsky, W. Role of AAA⁺-proteins in peroxisome biogenesis and function. *Biochim. Biophys. Acta - Mol. Cell Res.* **1863**, 828–837 (2016).
113. Lazarow, P. B. & Fujiki, Y. Biogenesis of peroxisomes. *Annu. Rev. Cell Biol.* **1**, 489–530 (1985).
114. Léon, S., Goodman, J. M. & Subramani, S. Uniqueness of the mechanism of protein import into the peroxisome matrix: Transport of folded, co-factor-bound and oligomeric proteins by shuttling receptors. *Biochimica et Biophysica Acta - Molecular Cell Research* **1763**, 1552–1564 (2006).

Bibliography

115. Veenhuis, M. & van der Klei, I. J. A critical reflection on the principles of peroxisome formation in yeast. *Frontiers in Physiology* **5 MAR**, (2014).
116. Knoops, K. *et al.* Preperoxisomal vesicles can form in the absence of Pex3. *J. Cell Biol.* **204**, 659–668 (2014).
117. Wróblewska, J. P. *et al.* *Saccharomyces cerevisiae* cells lacking Pex3 contain membrane vesicles that harbor a subset of peroxisomal membrane proteins. *Biochim. Biophys. Acta - Mol. Cell Res.* **1864**, 1656–1667 (2017).
118. Hettema, E. H., Erdmann, R., van der Klei, I. & Veenhuis, M. Evolving models for peroxisome biogenesis. *Current Opinion in Cell Biology* **29**, 25–30 (2014).
119. Sugiura, A., Mattie, S., Prudent, J. & McBride, H. M. Newly born peroxisomes are a hybrid of mitochondrial and ER-derived pre-peroxisomes. *Nature* **542**, 251–254 (2017).
120. Rucktäschel, R., Girzalsky, W. & Erdmann, R. Protein import machineries of peroxisomes. *Biochimica et Biophysica Acta - Biomembranes* **1808**, 892–900 (2011).
121. Gould, S. J., Keller, G. A. & Subramani, S. Identification of a peroxisomal targeting signal at the carboxy terminus of firefly luciferase. *J. Cell Biol.* **105**, 2923–2931 (1987).
122. Gould, S. J., Krisans, S., Keller, G. A. & Subramani, S. Antibodies directed against the peroxisomal targeting signal of firefly luciferase recognize multiple mammalian peroxisomal proteins. *J. Cell Biol.* **110**, 27–34 (1990).
123. Gould, S. J., Keller, G. A., Hosken, N., Wilkinson, J. & Subramani, S. A conserved tripeptide sorts proteins to peroxisomes. *J. Cell Biol.* **108**, 1657–1664 (1989).
124. Brocard, C. & Hartig, A. Peroxisome targeting signal 1: Is it really a simple tripeptide? *Biochimica et Biophysica Acta - Molecular Cell Research* **1763**, 1565–1573 (2006).
125. Brocard, C., Kragler, F., Simon, M. M., Schuster, T. & Hartig, A. The tetratricopeptide repeat-domain of the PAS10 protein of *Saccharomyces cerevisiae* is essential for binding the peroxisomal targeting signal-SKL. *Biochemical and*

- biophysical research communications* **204**, 1016–1022 (1994).
126. Stanley, W. A. *et al.* Recognition of a Functional Peroxisome Type 1 Target by the Dynamic Import Receptor Pex5p. *Mol. Cell* **24**, 653–663 (2006).
 127. Swinkels, B. W., Gould, S. J., Bodnar, A. G., Rachubinski, R. A. & Subramani, S. A novel, cleavable peroxisomal targeting signal at the amino-terminus of the rat 3-ketoacyl-CoA thiolase. *EMBO J.* **10**, 3255–62 (1991).
 128. Osumi, T. *et al.* Amino-terminal presequence of the precursor of peroxisomal 3-ketoacyl-CoA thiolase is a cleavable signal peptide for peroxisomal targeting. *Biochem. Biophys. Res. Commun.* **181**, 947–954 (1991).
 129. Helm, M. *et al.* Dual specificities of the glyoxysomal/peroxisomal processing protease Deg15 in higher plants. *Proc. Natl. Acad. Sci.* **104**, 11501–11506 (2007).
 130. Schuhmann, H., Huesgen, P. F., Gietl, C. & Adamska, I. The DEG15 serine protease cleaves peroxisomal targeting signal 2-containing proteins in Arabidopsis. *Plant Physiol.* **148**, 1847–1856 (2008).
 131. Kurochkin, I. V *et al.* Novel peroxisomal protease Tysnd1 processes PTS1- and PTS2-containing enzymes involved in beta-oxidation of fatty acids. *EMBO J.* **26**, 835–845 (2007).
 132. Rachubinski, R. A. & Subramani, S. How proteins penetrate peroxisomes. *Cell* **83**, 525–8 (1995).
 133. Rehling, P. *et al.* The import receptor for the peroxisomal targeting signal 2 (PTS2) in *Saccharomyces cerevisiae* is encoded by the PAS7 gene. *EMBO J.* **15**, 2901–13 (1996).
 134. Einwächter, H., Sowinski, S., Kunau, W. H. & Schliebs, W. *Yarrowia lipolytica* Pex20p, *Saccharomyces cerevisiae* Pex18p/Pex21p and mammalian Pex5pL fulfil a common function in the early steps of the peroxisomal PTS2 import pathway. *EMBO Rep.* **2**, 1035–1039 (2001).
 135. Glover, J. R., Andrews, D. W. & Rachubinski, R. A. *Saccharomyces cerevisiae* peroxisomal thiolase is imported as a dimer. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 10541–5 (1994).

136. Islinger, M., Li, K. W., Seitz, J., Völkl, A. & Lüers, G. H. Hitchhiking of Cu/Zn superoxide dismutase to peroxisomes - Evidence for a natural piggyback import mechanism in mammals. *Traffic* **10**, 1711–1721 (2009).
137. Klein, A. T. J., Van Den Berg, M. V., Bottger, G., Tabak, H. F. & Distel, B. *Saccharomyces cerevisiae* acyl-CoA oxidase follows a novel, non-PTS1, import pathway into peroxisomes that is dependent on Pex5p. *J. Biol. Chem.* **277**, 25011–25019 (2002).
138. Brown, L. A. & Baker, A. Shuttles and cycles: transport of proteins into the peroxisome matrix (review). *Mol Membr Biol* **25**, 363–375 (2008).
139. Albertini, M. *et al.* Pex14p, a Peroxisomal Membrane Protein Binding Both Receptors of the Two PTS-Dependent Import Pathways. *Cell* **89**, 83–92 (1997).
140. Rayapuram, N. & Subramani, S. The importomer-A peroxisomal membrane complex involved in protein translocation into the peroxisome matrix. *Biochimica et Biophysica Acta - Molecular Cell Research* **1763**, 1613–1619 (2006).
141. Erdmann, R. & Blobel, G. Identification of Pex13p, a peroxisomal membrane receptor for the PTS1 recognition factor. *J. Cell Biol.* **135**, 111–121 (1996).
142. Stein, K., Schell-Steven, A., Erdmann, R. & Rottensteiner, H. Interactions of Pex7p and Pex18p/Pex21p with the peroxisomal docking machinery: implications for the first steps in PTS2 protein import. *Mol. Cell. Biol.* **22**, 6056–69 (2002).
143. Miyata, N. & Fujiki, Y. Shuttling mechanism of peroxisome targeting signal type 1 receptor Pex5: ATP-independent import and ATP-dependent export. *Mol. Cell. Biol.* **25**, 10822–32 (2005).
144. Mukai, S. & Fujiki, Y. Molecular mechanisms of import of peroxisome-targeting signal type 2 (PTS2) proteins by PTS2 receptor Pex7p and PTS1 receptor Pex5pL. *J. Biol. Chem.* **281**, 37311–37320 (2006).
145. Grunau, S. *et al.* Peroxisomal targeting of PTS2 pre-import complexes in the yeast *saccharomyces cerevisiae*. *Traffic* **10**, 451–460 (2009).
146. Hasan, S., Platta, H. W. & Erdmann, R. Import of proteins into the peroxisomal matrix. *Frontiers in Physiology* **4 SEP**, (2013).

147. Dammai, V. & Subramani, S. The human peroxisomal targeting signal receptor, Pex5p, is translocated into the peroxisomal matrix and recycled to the cytosol. *Cell* **105**, 187–196 (2001).
148. Schnell, D. J. & Hebert, D. N. Protein translocons: Multifunctional mediators of protein translocation across membranes. *Cell* **112**, 491–505 (2003).
149. Erdmann, R. & Schliebs, W. Peroxisomal matrix protein import: the transient pore model. *Nat. Rev. Mol. Cell Biol.* **6**, 738–742 (2005).
150. Gouveia, A. M. M., Reguenga, C., Oliveira, M. E. M., Sa-Miranda, C. & Azevedo, J. E. Characterization of peroxisomal Pex5p from rat liver: Pex5p in the Pex5p-Pex14p membrane complex is a transmembrane protein. *J. Biol. Chem.* **275**, 32444–32451 (2000).
151. Ma, C., Schumann, U., Rayapuram, N. & Subramani, S. The Peroxisomal Matrix Import of Pex8p Requires Only PTS Receptors and Pex14p. *Mol. Biol. Cell* **20**, 3680–3689 (2009).
152. Platta, H. W., Grunau, S., Rosenkranz, K., Girzalsky, W. & Erdmann, R. Functional role of the AAA peroxins in dislocation of the cycling PTS1 receptor back to the cytosol. *Nat. Cell Biol.* **7**, 817–822 (2005).
153. Birschmann, I. *et al.* Pex15p of *Saccharomyces cerevisiae* provides a molecular basis for recruitment of the AAA peroxin Pex6p to peroxisomal membranes. *Mol. Biol. Cell* **14**, 2226–2236 (2003).
154. Matsumoto, N., Tamura, S. & Fujiki, Y. The pathogenic peroxin Pex26p recruits the Pex1p-Pex6p AAA ATPase complexes to peroxisomes. *Nat. Cell Biol.* **5**, 454–60 (2003).
155. Carvalho, A. F. *et al.* Ubiquitination of mammalian Pex5p, the peroxisomal import receptor. *J. Biol. Chem.* **282**, 31267–31272 (2007).
156. Gualdrón-López, M. *et al.* Ubiquitination of the glycosomal matrix protein receptor PEX5 in *Trypanosoma brucei* by PEX4 displays novel features. *Biochim. Biophys. Acta - Mol. Cell Res.* **1833**, 3076–3092 (2013).
157. Grou, C. P. *et al.* Members of the E2D (UbcH5) family mediate the ubiquitination of

- the conserved cysteine of Pex5p, the peroxisomal import receptor. *J. Biol. Chem.* **283**, 14190–14197 (2008).
158. Miyata, N., Okumoto, K., Mukai, S., Noguchi, M. & Fujiki, Y. AWP1/ZFAND6 functions in Pex5 export by interacting with cys-monoubiquitinated Pex5 and Pex6 AAA ATPase. *Traffic* **13**, 168–183 (2012).
159. El Magraoui, F. *et al.* The RING-type ubiquitin ligases Pex2p, Pex10p and Pex12p form a heteromeric complex that displays enhanced activity in an ubiquitin conjugating enzyme-selective manner. *FEBS J.* **279**, 2060–2070 (2012).
160. Komander, D., Clague, M. J. & Urbé, S. Breaking the chains: structure and function of the deubiquitinases. *Nat. Rev. Mol. Cell Biol.* **10**, 550–563 (2009).
161. Santos, M. J., Imanaka, T., Shio, H., Small, G. M. & Lazarow, P. B. Peroxisomal membrane ghosts in Zellweger syndrome--aberrant organelle assembly. *Science* **239**, 1536–8 (1988).
162. Hettema, E. H. *et al.* *Saccharomyces cerevisiae* pex3p and pex19p are required for proper localization and stability of peroxisomal membrane proteins. *EMBO J.* **19**, 223–33 (2000).
163. Mayerhofer, P. U. Targeting and insertion of peroxisomal membrane proteins: ER trafficking versus direct delivery to peroxisomes. *Biochim. Biophys. Acta - Mol. Cell Res.* **1863**, 870–880 (2016).
164. Aranovich, A., Hua, R., Rutenberg, A. D. & Kim, P. K. PEX16 contributes to peroxisome maintenance by constantly trafficking PEX3 via the ER. *J. Cell Sci.* **127**, 3675–3686 (2014).
165. Schrader, M. & Pellegrini, L. The making of a mammalian peroxisome, version 2.0: Mitochondria get into the mix. *Cell Death and Differentiation* **24**, 1148–1152 (2017).
166. Rottensteiner, H. *et al.* Peroxisomal membrane proteins contain common Pex19p-binding sites that are an integral part of their targeting signals. *Mol. Biol. Cell* **15**, 3406–3417 (2004).
167. Fang, Y., Morrell, J. C., Jones, J. M. & Gould, S. J. PEX3 functions as a PEX19

- docking factor in the import of class I peroxisomal membrane proteins. *J. Cell Biol.* **164**, 863–875 (2004).
168. Shibata, H., Kashiwayama, Y., Imanaka, T. & Kato, H. Domain architecture and activity of human Pex19p, a chaperone-like protein for intracellular trafficking of peroxisomal membrane proteins. *J. Biol. Chem.* **279**, 38486–38494 (2004).
169. Sacksteder, K. A. *et al.* PEX19 binds multiple peroxisomal membrane proteins, is predominantly cytoplasmic, and is required for peroxisome membrane synthesis. *J. Cell Biol.* **148**, 931–944 (2000).
170. Pinto, M. P. *et al.* The import competence of a peroxisomal membrane protein is determined by Pex19p before the docking step. *J. Biol. Chem.* **281**, 34492–34502 (2006).
171. Schmidt, F. *et al.* The Role of Conserved PEX3 Regions in PEX19-Binding and Peroxisome Biogenesis. *Traffic* **13**, 1244–1260 (2012).
172. Hoepfner, D., Schildknegt, D., Braakman, I., Philippsen, P. & Tabak, H. F. Contribution of the endoplasmic reticulum to peroxisome formation. *Cell* **122**, 85–95 (2005).
173. Sato, Y. *et al.* Structural basis for docking of peroxisomal membrane protein carrier Pex19p onto its receptor Pex3p. *EMBO J.* **29**, 4083–93 (2010).
174. Schmidt, F. *et al.* Insights into peroxisome function from the structure of PEX3 in complex with a soluble fragment of PEX19. *J. Biol. Chem.* **285**, 25410–25417 (2010).
175. Pinto, M. P., Grou, C. P., Fransen, M., Sá-Miranda, C. & Azevedo, J. E. The cytosolic domain of PEX3, a protein involved in the biogenesis of peroxisomes, binds membrane lipids. *Biochim. Biophys. Acta - Mol. Cell Res.* **1793**, 1669–1675 (2009).
176. Fagarasanu, M., Fagarasanu, A., Tam, Y. Y. C., Aitchison, J. D. & Rachubinski, R. A. Inp1p is a peroxisomal membrane protein required for peroxisome inheritance in *Saccharomyces cerevisiae*. *J. Cell Biol.* **169**, 765–775 (2005).
177. Novikoff, A. B., Novikoff, P. M., Davis, C. & Quintana, N. Studies on

- Microperoxisomes - II. A Cytochemical Method for Light and Electron Microscopy. *J. Histochem. Cytochem.* **20**, 1006–1023 (1972).
178. Fujiki, Y., Rachubinski, R. A. & Lazarow, P. B. Synthesis of a major integral membrane polypeptide of rat liver peroxisomes on free polysomes. *Proc. Natl. Acad. Sci. U. S. A.* **81**, 7127–31 (1984).
 179. Höhfeld, J., Veenhuis, M. & Kunau, W. H. PAS3, a *Saccharomyces cerevisiae* gene encoding a peroxisomal integral membrane protein essential for peroxisome biogenesis. *J. Cell Biol.* **114**, 1167–78 (1991).
 180. Baerends, R. J. S. *et al.* The *Hansenula polymorpha* PER9 gene encodes a peroxisomal membrane protein essential for peroxisome assembly and integrity. *J. Biol. Chem.* **271**, 8887–8894 (1996).
 181. Titorenko, V. I. & Rachubinski, R. a. Mutants of the yeast *Yarrowia lipolytica* defective in protein exit from the endoplasmic reticulum are also defective in peroxisome biogenesis. *Mol. Cell. Biol.* **18**, 2789–803 (1998).
 182. Jones, J. M., Morrell, J. C. & Gould, S. J. PEX19 is a predominantly cytosolic chaperone and import receptor for class 1 peroxisomal membrane proteins. *J. Cell Biol.* **164**, 57–67 (2004).
 183. Fujiki, Y., Matsuzono, Y., Matsuzaki, T. & Fransen, M. Import of peroxisomal membrane proteins: The interplay of Pex3p- and Pex19p-mediated interactions. *Biochimica et Biophysica Acta - Molecular Cell Research* **1763**, 1639–1646 (2006).
 184. Halbach, A., Rucktäschel, R., Rottensteiner, H. & Erdmann, R. The N-domain of Pex22p can functionally replace the Pex3p N-domain in targeting and peroxisome formation. *J. Biol. Chem.* **284**, 3906–3916 (2009).
 185. Mayerhofer, P. U., Bañó-Polo, M., Mingarro, I. & Johnson, A. E. Human Peroxin PEX3 Is Co-translationally Integrated into the ER and Exits the ER in Budding Vesicles. *Traffic* **17**, 117–130 (2016).
 186. Kragt, A., Voorn-Brouwer, T., Van Den Berg, M. & Distel, B. Endoplasmic reticulum-directed Pex3p routes to peroxisomes and restores peroxisome formation in a *Saccharomyces cerevisiae* pex3?? strain. *J. Biol. Chem.* **280**, 34350–34357

- (2005).
187. Rucktäschel, R., Halbach, A., Girzalsky, W., Rottensteiner, H. & Erdmann, R. De novo synthesis of peroxisomes upon mitochondrial targeting of Pex3p. *Eur. J. Cell Biol.* **89**, 947–954 (2010).
 188. Thoms, S., Harms, I., Kalies, K. U. & Gärtner, J. Peroxisome Formation Requires the Endoplasmic Reticulum Channel Protein Sec61. *Traffic* **13**, 599–609 (2012).
 189. Young, B. P., Craven, R. A., Reid, P. J., Willer, M. & Stirling, C. J. Sec63p and Kar2p are required for the translocation of SRP-dependent precursors into the yeast endoplasmic reticulum in vivo. *EMBO J.* **20**, 262–271 (2001).
 190. Okreglak, V. & Walter, P. The conserved AAA-ATPase Msp1 confers organelle specificity to tail-anchored proteins. *Proc. Natl. Acad. Sci.* **111**, 8019–8024 (2014).
 191. Halbach, A. Targeting of the tail-anchored peroxisomal membrane proteins PEX26 and PEX15 occurs through C-terminal PEX19-binding sites. *J. Cell Sci.* **119**, 2508–2517 (2006).
 192. Chen, Y. *et al.* Hydrophobic handoff for direct delivery of peroxisome tail-anchored proteins. *Nat. Commun.* **5**, 5790 (2014).
 193. Delille, H. K. & Schrader, M. Targeting of hFis1 to peroxisomes is mediated by Pex19p. *J. Biol. Chem.* **283**, 31107–31115 (2008).
 194. Conibear, E. & Stevens, T. H. Studying yeast vacuoles. *Methods in Enzymology* **351**, 408–432 (2002).
 195. Alberts, B. *et al.* *Molecular Biology of the Cell*, 4th edition. Garland Science (2002). doi:10.3389/fimmu.2015.00171
 196. Booth, A. & Doolittle, W. F. Eukaryogenesis, how special really? *Proc. Natl. Acad. Sci.* **112**, 10278–10285 (2015).
 197. Blackstone, N. An Evolutionary Framework for Understanding the Origin of Eukaryotes. *Biology (Basel)*. **5**, 18 (2016).
 198. Fukasawa, Y., Oda, T., Tomii, K. & Imai, K. Origin and Evolutionary Alteration of the Mitochondrial Import System in Eukaryotic Lineages. *Mol. Biol. Evol.* **34**, 1574–1586 (2017).

199. Derelle, R. *et al.* Bacterial proteins pinpoint a single eukaryotic root. *Proc. Natl. Acad. Sci.* **112**, E693–E699 (2015).
200. Mackiewicz, P. & Gagat, P. Monophyly of Archaeplastida supergroup and relationships among its lineages in the light of phylogenetic and phylogenomic studies. Are we close to a consensus? *Acta Societatis Botanicorum Poloniae* **83**, 263–280 (2014).
201. Torruella, G. *et al.* Phylogenetic relationships within the Opisthokonta based on phylogenomic analyses of conserved single-copy protein domains. *Mol. Biol. Evol.* **29**, 531–544 (2012).
202. Gould, S. B., Garg, S. G. & Martin, W. F. Bacterial Vesicle Secretion and the Evolutionary Origin of the Eukaryotic Endomembrane System. *Trends in Microbiology* **24**, 525–534 (2016).
203. Lucattini, R., Likić, V. A. & Lithgow, T. Bacterial Proteins Predisposed for Targeting to Mitochondria. *Mol. Biol. Evol.* **21**, 652–658 (2004).
204. Mani, J., Meisinger, C. & Schneider, A. Peeping at TOMs - Diverse entry gates to mitochondria provide insights into the evolution of eukaryotes. *Mol. Biol. Evol.* **33**, 337–351 (2016).
205. Mani, J. *et al.* Mitochondrial protein import receptors in Kinetoplastids reveal convergent evolution over large phylogenetic distances. *Nat. Commun.* **6**, 6646 (2015).
206. Mani, J., Rout, S., Desy, S. & Schneider, A. Mitochondrial protein import - Functional analysis of the highly diverged Tom22 orthologue of *Trypanosoma brucei*. *Sci. Rep.* **7**, 40738 (2017).
207. Perry, A. J., Hulett, J. M., Likić, V. A., Lithgow, T. & Gooley, P. R. Convergent evolution of receptors for protein import into mitochondria. *Curr. Biol.* **16**, 221–229 (2006).
208. Schatz, G. Just follow the acid chain. *Nature* **388**, 121–122 (1997).
209. Singha, U. K. *et al.* Characterization of the mitochondrial inner membrane protein translocator Tim17 from *Trypanosoma brucei*. *Mol. Biochem. Parasitol.* **159**, 30–43

- (2008).
210. Žárský, V. & Doležal, P. Evolution of the Tim17 protein family. *Biol. Direct* **11**, 54 (2016).
 211. Clements, A. *et al.* The reducible complexity of a mitochondrial molecular machine. *Proc. Natl. Acad. Sci.* **106**, 15791–15795 (2009).
 212. Youngman, M. J., Hobbs, A. E. A., Burgess, S. M., Srinivasan, M. & Jensen, R. E. Mmm2p, a mitochondrial outer membrane protein required for yeast mitochondrial shape and maintenance of mtDNA nucleoids. *J. Cell Biol.* **164**, 677–688 (2004).
 213. Burri, L. & Lithgow, T. A complete set of SNAREs in yeast. *Traffic* **5**, 45–52 (2004).
 214. Verner, K. Co-translational protein import into mitochondria: an alternative view. *Trends Biochem. Sci.* **18**, 366–371 (1993).
 215. Yoshida, H. *et al.* YqjD is an inner membrane protein associated with stationary-phase ribosomes in *Escherichia coli*. *J. Bacteriol.* **194**, 4178–4183 (2012).
 216. Harris, N. J. *et al.* Structure formation during translocon-unassisted co-translational membrane protein folding. *Sci. Rep.* **7**, 8021 (2017).
 217. Gurevich, V. V, Pokrovskaya, I. D., Garnovskaya, M. N., Dumler, I. L. & Zozulya, S. A. Proper cotranslational insertion of visual rhodopsin into the lipid bilayer occurs in the absence of protein translocation machinery. *Biomed Sci* **2**, 187–192 (1991).
 218. Lemire, B. D., Fankhauser, C., Baker, A. & Schatz, G. The mitochondrial targeting function of randomly generated peptide sequences correlates with predicted helical amphiphilicity. *J. Biol. Chem.* **264**, 20206–20215 (1989).
 219. Kim, S., Lee, H. & Park, S. The Structure of *Escherichia coli* TcdA (Also Known As CsdL) Reveals a Novel Topology and Provides Insight into the tRNA Binding Surface Required for N6-Threonylcarbamoyladenine Dehydratase Activity. *J. Mol. Biol.* **427**, 3074–3085 (2015).
 220. Duve, C. de. PEROXISOMES AND RELATED PARTICLES IN HISTORICAL PERSPECTIVE. *Ann. N. Y. Acad. Sci.* **386**, 1–4 (1982).

221. Gabaldon, T. Peroxisome diversity and evolution. *Philos. Trans. R. Soc. B Biol. Sci.* **365**, 765–773 (2010).
222. Speijer, D. Evolution of peroxisomes illustrates symbiogenesis. *BioEssays* **39**, (2017).
223. Liu, Y., Fiskum, G. & Schubert, D. Generation of reactive oxygen species by the mitochondrial electron transport chain. *J Neurochem* **80**, 780–787 (2002).
224. Guo, Y. *et al.* Tail-anchored inner membrane protein ElaB increases resistance to stress while reducing persistence in Escherichia coli. *J. Bacteriol.* **199**, (2017).
225. Villanueva, L., Schouten, S. & Damsté, J. S. S. Phylogenomic analysis of lipid biosynthetic genes of Archaea shed light on the ‘lipid divide’. *Environ. Microbiol.* **19**, 54–69 (2017).
226. Walther, D. M., Papic, D., Bos, M. P., Tommassen, J. & Rapaport, D. Signals in bacterial beta-barrel proteins are functional in eukaryotic cells for targeting to and assembly in mitochondria. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 2531–2536 (2009).
227. Koonin, E. V., Makarova, K. S. & Aravind, L. Horizontal Gene Transfer in Prokaryotes: Quantification and Classification. *Annu. Rev. Microbiol.* **55**, 709–742 (2001).
228. Keeling, P. J. & Palmer, J. D. Horizontal gene transfer in eukaryotic evolution. *Nat. Rev. Genet.* **9**, 605–618 (2008).
229. Garipler, G., Mutlu, N., Lack, N. A. & Dunn, C. D. Deletion of conserved protein phosphatases reverses defects associated with mitochondrial DNA damage in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 1473–8 (2014).
230. Mutlu, N., Garipler, G., Akdoğan, E. & Dunn, C. D. Activation of the Pleiotropic Drug Resistance Pathway Can Promote Mitochondrial DNA Retention by Fusion-Defective Mitochondria in *Saccharomyces cerevisiae*. *G3 (Bethesda)*. **4**, 1247–1258 (2014).
231. Sikorski, R. S. & Hieter, P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19–27 (1989).

232. Prinz, W. A. *et al.* Mutants affecting the structure of the cortical endoplasmic reticulum in *Saccharomyces cerevisiae*. *J. Cell Biol.* **150**, 461–474 (2000).
233. Ryan, K. R., Leung, R. S. & Jensen, R. E. Characterization of the mitochondrial inner membrane translocase complex: the Tim23p hydrophobic domain interacts with Tim17p but not with other Tim23p molecules. *Mol. Cell. Biol.* **18**, 178–87 (1998).
234. Taxis, C. & Knop, M. System of centromeric, episomal, and integrative vectors based on drug resistance markers for *Saccharomyces cerevisiae*. *Biotechniques* **40**, 73–78 (2006).
235. Lee, S., Lim, W. A. & Thorn, K. S. Improved Blue, Green, and Red Fluorescent Protein Tagging Vectors for *S. cerevisiae*. *PLoS One* **8**, (2013).
236. Lazarow, P. B. & De Duve, C. A fatty acyl-CoA oxidizing system in rat liver peroxisomes; enhancement by clofibrate, a hypolipidemic drug. *Proc. Natl. Acad. Sci. U. S. A.* **73**, 2043–6 (1976).
237. De Duve, C. & Baudhuin, P. Peroxisomes (microbodies and related particles). *Physiol. Rev.* **46**, 323–357 (1966).
238. Subramani, S. Components involved in peroxisome import, biogenesis, proliferation, turnover, and movement. *Physiol. Rev.* **78**, 171–188 (1998).
239. Steinberg, S. J. *et al.* Peroxisome biogenesis disorders. *Biochimica et Biophysica Acta - Molecular Cell Research* **1763**, 1733–1748 (2006).
240. Krogh, a, Larsson, B., von Heijne, G. & Sonnhammer, E. L. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J. Mol. Biol.* **305**, 567–580 (2001).
241. Weir, N. R., Kamber, R. A., Martenson, J. S. & Denic, V. The AAA protein Msp1 mediates clearance of excess tail-anchored proteins from the peroxisomal membrane. *Elife* (2017).
242. Rhodin, J. Correlation of ultrastructural organization and function in normal and experimentally changed proximal tubule cells of the mouse kidney. (Karonlinska Institutet, 1954).

243. Maddalo, G. *et al.* Systematic analysis of native membrane protein complexes in *Escherichia coli*. *J. Proteome Res.* **10**, 1848–1859 (2011).
244. Cavalier-Smith, T. Cell and genome coevolution: Facultative anaerobiosis, glycosomes and kinetoplast RNA editing. *Trends in Genetics* **13**, 6–9 (1997).
245. Kalish, J. E. *et al.* Characterization of a novel component of the peroxisomal protein import apparatus using fluorescent peroxisomal proteins. *Embo J.* **15**, 3275–3285 (1996).
246. Dunn, C. D. & Jensen, R. E. Suppression of a defect in mitochondrial protein import identifies cytosolic proteins required for viability of yeast cells lacking mitochondrial DNA. *Genetics* **165**, 35–45 (2003).
247. Garipler, G. & Dunn, C. D. Defects associated with mitochondrial DNA damage can be mitigated by increased vacuolar pH in *Saccharomyces cerevisiae*. *Genetics* **194**, 285–290 (2013).
248. Örs, Ş. T., Akdoğan, E. & Dunn, C. D. Mutation of the mitochondrial large ribosomal RNA can provide pentamidine resistance to *Saccharomyces cerevisiae*. *Mitochondrion* **18**, 7–11 (2014).
249. Sesaki, H. & Jensen, R. E. Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape. *J. Cell Biol.* **147**, 699–706 (1999).
250. Metzger, M. B., Maurer, M. J., Dancy, B. M. & Michaelis, S. Degradation of a cytosolic protein requires endoplasmic reticulum-associated degradation machinery. *J. Biol. Chem.* **283**, 32302–32316 (2008).
251. DeLoache, W. C., Russ, Z. N. & Dueber, J. E. Towards repurposing the yeast peroxisome for compartmentalizing heterologous metabolic pathways. *Nat. Commun.* **7**, 11152 (2016).
252. Osellame, L. D., Blacker, T. S. & Duchen, M. R. Cellular and molecular mechanisms of mitochondrial function. *Best Pract. Res. Clin. Endocrinol. Metab.* **26**, 711–23 (2012).
253. Rouault, T. A. Biogenesis of iron-sulfur clusters in mammalian cells: new insights and relevance to human disease. *Dis Model Mech* **5**, 155–164 (2012).

254. Susin, S. a *et al.* Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**, 441–446 (1999).
255. Nicolson, G. L. Mitochondrial dysfunction and chronic disease: treatment with natural supplements. *Altern. Ther. Health Med.* **20 Suppl 1**, 18–25 (2014).
256. Boland, M. L., Chourasia, A. H. & Macleod, K. F. Mitochondrial Dysfunction in Cancer. *Front. Oncol.* **2**, 292 (2013).
257. Schmidt, O., Pfanner, N. & Meisinger, C. Mitochondrial protein import: from proteomics to functional mechanisms. *Nat Rev Mol Cell Biol* **11**, (2010).
258. MacKenzie, J. A. & Payne, M. R. Mitochondrial Protein Import and Human Health and Disease. *Biochim Biophys Acta* **1772**, 509–523 (2007).
259. Hoogenraad, N. J., Ward, L. A. & Ryan, M. T. Import and assembly of proteins into mitochondria of mammalian cells. *Biochimica et Biophysica Acta - Molecular Cell Research* **1592**, 97–105 (2002).
260. Mihara, K. & Omura, T. Cytosolic factors in mitochondrial protein import. *Experientia* **52**, 1063–1068 (1996).
261. Murakami, K. & Mori, M. Purified presequence binding factor (PBF) forms an import-competent complex with a purified mitochondrial precursor protein. *EMBO J.* **9**, 3201–3208 (1990).
262. Komiya, T., Rospert, S., Schatz, G. & Mihara, K. Binding of mitochondrial precursor proteins to the cytoplasmic domains of the import receptors Tom70 and Tom20 is determined by cytoplasmic chaperones. *EMBO J.* **16**, 4267–4275 (1997).
263. Seki, N. *et al.* A human homolog of the mitochondrial protein import receptor Mom19 can assemble with the yeast mitochondrial receptor complex. *FEBS Lett.* **375**, 307–310 (1995).
264. Goping, I. S., Millar, D. G. & Shore, G. C. Identification of the human mitochondrial protein import receptor, huMas20p. Complementation of delta mas20 in yeast. *FEBS Lett.* **373**, 45–50 (1995).
265. Abe, Y. *et al.* Structural basis of presequence recognition by the mitochondrial import receptor Tom20. *Cell* **100**, 551–560 (2000).

Bibliography

266. Eliyahu, E. *et al.* Tom20 Mediates Localization of mRNAs to Mitochondria in a Translation-Dependent Manner. *Mol. Cell. Biol.* **30**, 284–294 (2010).
267. Lesnik, C., Golani-Armon, A. & Arava, Y. Localized translation near the mitochondrial outer membrane: An update. *RNA Biol.* **12**, 801–809 (2015).
268. Gehrke, S. *et al.* PINK1 and parkin control localized translation of respiratory chain component mRNAs on mitochondria outer membrane. *Cell Metab.* **21**, 95–108 (2015).
269. Yano, M. *et al.* Visualization of mitochondrial protein import in cultured mammalian cells with green fluorescent protein and effects of overexpression of the human import receptor Tom20. *J. Biol. Chem.* **272**, 8459–8465 (1997).
270. Harkness, T. A. A., Nargang, F. E., Van Der Klei, I., Neupert, W. & Lill, R. A crucial role of the mitochondrial protein import receptor MOM19 for the biogenesis of mitochondria. *J. Cell Biol.* **124**, 637–648 (1994).
271. Bolliger, L., Junne, T., Schatz, G. & Lithgow, T. Acidic receptor domains on both sides of the outer membrane mediate translocation of precursor proteins into yeast mitochondria. *EMBO J.* **14**, 6318–6326 (1995).
272. Yano, M., Hoogenraad, N., Terada, K. & Mori, M. Identification and functional analysis of human Tom22 for protein import into mitochondria. *Mol. Cell. Biol.* **20**, 7205–13 (2000).
273. Suzuki, H. *et al.* Characterization of rat TOM40, a central component of the preprotein translocase of the mitochondrial outer membrane. *J. Biol. Chem.* **275**, 37930–37936 (2000).
274. Mokranjac, D. Mitochondrial protein import: An unexpected disulfide bond. *J. Cell Biol.* **214**, 363–365 (2016).
275. Bauer, M. F. *et al.* Genetic and structural characterization of the human mitochondrial inner membrane translocase. *J. Mol. Biol.* **289**, 69–82 (1999).
276. Rainbolt, T. K., Atanassova, N., Genereux, J. C. & Wiseman, R. L. Stress-regulated translational attenuation adapts mitochondrial protein import through Tim17A degradation. *Cell Metab.* **18**, 908–919 (2013).

Bibliography

277. Blom, J. *et al.* The essential yeast protein MIM44 (encoded by MPI1) is involved in an early step of preprotein translocation across the mitochondrial inner membrane. *Mol. Cell. Biol.* **13**, 7364–71 (1993).
278. Ishihara, N. & Mihara, K. Identification of the protein import components of the rat mitochondrial inner membrane, rTIM17, rTIM23, and rTIM44. *J. Biochem.* **123**, 722–732 (1998).
279. Moro, F., Okamoto, K., Donzeau, M., Neupert, W. & Brunner, M. Mitochondrial protein import: Molecular basis of the ATP-dependent interaction of MtHsp70 with Tim44. *J. Biol. Chem.* **277**, 6874–6880 (2002).
280. Bauer, M. F. *et al.* The mitochondrial TIM22 preprotein translocase is highly conserved throughout the eukaryotic kingdom. *FEBS Lett.* **464**, 41–47 (1999).
281. Callegari, S. *et al.* TIM29 is a subunit of the human carrier translocase required for protein transport. *FEBS Letters* **590**, 4147–4158 (2016).
282. Chew, a, Sirugo, G., Alsobrook, J. P. & Isaya, G. Functional and genomic analysis of the human mitochondrial intermediate peptidase, a putative protein partner of frataxin. *Genomics* **65**, 104–12 (2000).
283. Corydon, T. J., Hansen, J., Bross, P. & Jensen, T. G. Down-regulation of Hsp60 expression by RNAi impairs folding of medium-chain acyl-CoA dehydrogenase wild-type and disease-associated proteins. *Mol. Genet. Metab.* **85**, 260–270 (2005).
284. Legname, G. *et al.* Expression in *Escherichia coli*, purification and functional activity of recombinant human chaperonin 10. *FEBS Lett.* **361**, 211–214 (1995).
285. Bhattacharyya, T. *et al.* Cloning and subcellular localization of human mitochondrial hsp70. *J. Biol. Chem.* **270**, 1705–1710 (1995).
286. Harrison, C. GrpE, a nucleotide exchange factor for DnaK. *Cell Stress Chaperones* **8**, 218 (2003).
287. Hirai, K. *et al.* Mitochondrial abnormalities in Alzheimer's disease. *J. Neurosci.* **21**, 3017–23 (2001).
288. Anandatheerthavarada, H. K., Biswas, G., Robin, M. A. & Avadhani, N. G. Mitochondrial targeting and a novel transmembrane arrest of Alzheimer's amyloid

- precursor protein impairs mitochondrial function in neuronal cells. *J. Cell Biol.* **161**, 41–54 (2003).
289. Moreira, P. I., Carvalho, C., Zhu, X., Smith, M. A. & Perry, G. Mitochondrial dysfunction is a trigger of Alzheimer's disease pathophysiology. *Biochim. Biophys. Acta - Mol. Basis Dis.* **1802**, 2–10 (2010).
 290. Madamba, S. M., Damri, K. N., Dejean, L. M. & Peixoto, P. M. Mitochondrial Ion Channels in Cancer Transformation. *Front. Oncol.* **5**, 120 (2015).
 291. Handa, N. *et al.* Structure of the human Tim44 C-terminal domain in complex with pentaethylene glycol: Ligand-bound form. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **63**, 1225–1234 (2007).
 292. Bellot, G. *et al.* TOM22, a core component of the mitochondria outer membrane protein translocation pore, is a mitochondrial receptor for the proapoptotic protein Bax. *Cell Death Differ.* **14**, 785–794 (2007).
 293. Kuwana, T. *et al.* Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* **111**, 331–342 (2002).
 294. Yano, M., Terada, K. & Mori, M. AIP is a mitochondrial import mediator that binds to both import receptor Tom20 and preproteins. *J. Cell Biol.* **163**, 45–56 (2003).
 295. Deshaies, R. J. & Schekman, R. A yeast mutant defective at an early stage in import of secretory protein precursors into the endoplasmic reticulum. *J. Cell Biol.* **105**, 633–645 (1987).
 296. Dekker, P. J. T. *et al.* Identification of MIM23, a putative component of the protein import machinery of the mitochondrial inner membrane. *FEBS Lett.* **330**, 66–70 (1993).
 297. Blochlinger, K. & Diggelmann, H. Hygromycin B phosphotransferase as a selectable marker for DNA transfer experiments with higher eucaryotic cells. *Mol. Cell. Biol.* **4**, 2929–2931 (1984).
 298. Zhang, G., Gurtu, V. & Kain, S. R. An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells. *Biochem. Biophys. Res. Commun.* **227**, 707–711 (1996).

299. Charrier, S. *et al.* Quantification of lentiviral vector copy numbers in individual hematopoietic colony-forming cells shows vector dose-dependent effects on the frequency and level of transduction. *Gene Ther.* **18**, 479–487 (2011).
300. Schatz, G. The Protein Import System of Mitochondria. *J. Biol. Chem.* **271**, 31763–31766 (1996).
301. Pittenger, R. *et al.* Hygromycin. I. Preliminary studies on the production and biologic activity of a new antibiotic. *Antibiot. Chemother.* **3**, 1268–1278 (1953).
302. van Meer, G., Voelker, D. R. & Feigenson, G. W. Membrane lipids: where they are and how they behave. *Nat. Rev. Mol. Cell Biol.* **9**, 112–124 (2008).
303. Yoon, Y. G. & Koob, M. D. Selection by drug resistance proteins located in the mitochondria of mammalian cells. *Mitochondrion* **8**, 345–351 (2008).
304. Mali, P. *et al.* RNA-guided human genome engineering via Cas9. *Science* **339**, 823–6 (2013).
305. Larson, M. H. *et al.* CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nat. Protoc.* **8**, 2180–2196 (2013).
306. Zhao, Q. *et al.* A mitochondrial specific stress response in mammalian cells. *EMBO J.* **21**, 4411–4419 (2002).
307. Robinson, D. A. *et al.* A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat. Genet.* **33**, 401–406 (2003).
308. Bratic, I. & Trifunovic, A. Mitochondrial energy metabolism and ageing. *Biochim. Biophys. Acta - Bioenerg.* **1797**, 961–967 (2010).
309. Weinberg, J. M., Venkatachalam, M. a, Roeser, N. F. & Nissim, I. Mitochondrial dysfunction during hypoxia/reoxygenation and its correction by anaerobic metabolism of citric acid cycle intermediates. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 2826–2831 (2000).
310. Tantama, M., Martínez-François, J. R., Mongeon, R. & Yellen, G. Imaging energy status in live cells with a fluorescent biosensor of the intracellular ATP-to-ADP ratio. *Nat. Commun.* **4**, (2013).

Bibliography

311. Aguer, C. *et al.* Galactose enhances oxidative metabolism and reveals mitochondrial dysfunction in human primary muscle cells. *PLoS One* **6**, (2011).
312. Wyatt, C. N. & Buckler, K. J. The effect of mitochondrial inhibitors on membrane currents in isolated neonatal rat carotid body type I cells. *J. Physiol.* **556**, 175–191 (2004).



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