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INTRACELLULAR TARGETING
OF TAIL-ANCHORED PROTEINS

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Tesi di Dottorato di:
BRUNA GUIOMAR FIGUEIREDO COSTA

Tutor: Prof. ALBERTO EMILIO PANERAI
Co-Tutors: Prof.ssa NICA BORGESE e Dott.ssa SARA COLOMBO

Coordinatore del Dottorato: Prof. ALBERTO CORSINI

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1. Abstract

1. Abstract

Le proteine con ancora carbossi-terminale, chiamate *tail-anchored* (proteine TA), costituiscono una classe di proteine di membrana coinvolte in funzioni fisiologiche fondamentali, quali la regolazione della esocitosi e dell'apoptosi. Le proteine TA hanno in comune una particolare topologia, che consiste in un dominio citosolico amino-terminale ancorata al doppio strato fosfolipidico da un segmento idrofobico molto vicino all'estremità carbossi-terminale. A causa di questa particolare topologia, queste proteine sono indirizzate alla loro destinazione mediante meccanismi esclusivamente post-traduzionali. Una via di *targeting* importante è centrata sulla ATPasi TRC40/Get3; tuttavia, almeno in condizioni acellulari, alcune proteine TA possono inserirsi in membrana in modo indipendente da questa via, e possono addirittura inserirsi spontaneamente in doppi strati fosfolipidici privi di proteine, senza assistenza di chaperone. Un esempio conosciuto di una proteina TA a inserzione spontanea è dato dal citocromo b5, una proteina coinvolta nel metabolismo di lipidi e xenobiotici. Sono conosciute due forme del citocromo b5, che *in vivo* si localizzano al Reticolo Endoplasmatico (b5-ER) oppure alla membrana esterna mitocondriale (b5-RR). Il problema affrontato nella mia tesi è di come sia raggiunta la specifica localizzazione *in vivo* delle due forme nonostante la loro promiscuità *in vitro.* Per studiare questo problema, ho messo a punto un sistema basato su cellule coltivate semi-intatte, che possono essere manipolate e analizzate con metodi biochimici e di microscopia. In presenza di citosol, sia b5-RR che b5-ER, aggiunti o come proteine ricombinanti oppure come prodotti tradotti *in vitro* erano consegnati fedelmente alla corretta destinazione. Invece, in assenza di citosol, ambedue si localizzavano ai mitocondri, a indicare che fattori citosolici sono richiesti per evitare la localizzazione sbagliata di b5-ER alla membrana esterna mitocondriale. Ho dimostrato anche che il *targeting* di b5-ER richiede energia, e che sia la via TRC40 che la via di *targeting* SND2 identificata recentemente sono coinvolte solamente in piccola parte. Per identificare altre vie, ho utilizzato una varietà di inibitori di basso peso molecolare, incluse molecole che inibiscono proteine *heat shock* e l'AAA-ATPasi p97. L'inibizione di Hsc70 e Hsp90 non ha avuto effetto, mente la Eeyarestatin (ES I), un inibitore sia di p97 che di Sec61 (il traslocone responsabile per la traslocazione co-traduzionale) ha ridotto efficacemente l'inserzione di b5-ER ma non di un substrato (sinaptobrevina 2) della via TRC40. Analogamente, ho trovato che il silenziamento di Sec61 ha inibito l'inserzione di b5-ER ma non di sinaptobrevina 2, a suggerire che l'interferenza di ES I è legata alla sua azione sul traslocone. Anche quando le due vie Sec61 e TRC40 sono state silenziate assieme, una notevole proporzione di b5-ER ha raggiunto il reticolo endoplasmatico, a indicare l'esistenza di altre vie di inserzione che rimangono da identificare. I miei risultati indicano che la membrana esterna mitocondriale rappresenta la via *default* per le proteine TA e rivelano l'esistenza di molteplici vie di *targeting* al reticolo, vie che sono ridondanti ma specifiche per substrati diversi.

1. Abstract

Tail-anchored (TA) proteins constitute a class of membrane proteins whose diverse members carry out basic functions in cell physiology, including regulation of exocytosis and of apoptosis. TA proteins share a particular topology, consisting in a cytosolically located N-terminal domain anchored to the bilayer by a C-terminal hydrophobic stretch. Because of this particular topology, these proteins are targeted to their destination by unique post-translational pathways. An important identified pathway is centred on the ATPase TRC40/Get3, however, at least under cell-free conditions, some TA proteins can access membranes independently from this pathway, and can even insert spontaneously into protein-free phospholipid bilayers without assistance from any chaperone. One example of a spontaneously inserting TA protein is cytochrome b5, a protein involved in lipid and drug metabolism. Two forms of cyt b5 are known, which *in* vivo target either the Endoplasmic Reticulum (b5-ER) or the mitochondrial outer membrane (b5-RR). The question addressed in my thesis is how *in* vivo specificity is attained in the face of *in* vitro promiscuity. To investigate this problem, I set up a system based on semi-intact cultured cells, which can be manipulated and analysed biochemically and by immunofluorescence. In the presence of cytosol, both b5-RR and b5-ER, added either as recombinant proteins or as *in vitro* translated products, were faithfully targeted to their correct destinations. In contrast, in the absence of cytosol, both forms targeted the mitochondria, indicating that cytosolic factors are required to avoid mislocalisation of b5-ER to the mitochondrial outer membrane. I further demonstrated that ER targeting is energy-dependent, and that both the TRC40 and the recently described SND2 pathways are minimally involved. To elucidate further pathways, I used a number of small molecule inhibitors, including ones that target heat shock proteins and the AAA-ATPase p97. Hsc70 and Hsp90 inhibition had no effect, whereas Eeyarestatin (ES I), an inhibitor of both p97 and Sec61 (the translocon responsible for co-translational translocation), strongly reduced insertion of b5-ER, but not of the TRC40 substrate Synaptobrevin 2. Similarly, I found that downregulation of Sec61, while having no effect on Synaptobrevin, inhibited b5-ER insertion, suggesting that ES I is acting by blocking the translocon. Even when Sec61 and the TRC40 pathway were blocked together, however, a large proportion of b5-ER still reached the ER, indicating the existence of yet additional pathways. My results indicate that the mitochondrial outer membrane represents the default destination of TA proteins, and reveal the existence of multiple, redundant, but substrate-specific ER targeting pathways.

2. Introduction

2.1 Membrane Proteins

Membrane proteins, which comprise ~30% of the proteome, are proteins that interact with, or are part of, biological membranes. Their efficient and accurate localisation is crucial for the structure and function of all cells, since they can be involved in signalling, intracellular trafficking, organelle biogenesis, and transport of a variety of molecules and ions across cellular membranes. Indeed, membrane proteins are targets of over 50% of all modern medicinal drugs (Shao and Hegde, 2011; Alberts *et al.*, 2014).

Many membrane proteins extend through the lipid bilayer, with part of their mass on either side (1,2 and 3 in Cartoon 1). These transmembrane proteins are amphipathic, i.e., they have both hydrophobic and hydrophilic parts. There are also membrane proteins completely localised in the cytosol; in this case, they can be associated by an amphipathic α-helix or by one or more covalently attached lipid chains (examples 4 and 5, respectively). The example 6 refers to membrane proteins that are entirely exposed outside of the cell, and that can be associated with the membrane only by a covalent linkage to phosphatidylinositol.

Some membrane proteins, like the ones in examples 7 and 8, are called peripheral membrane proteins, since they are bound to the membrane by non-covalent interactions with other membrane proteins. Because of this characteristic, peripheral membrane proteins can be extracted from the membrane by gentle extraction procedures (for example solutions of very high or low ionic strength or of extreme pH). All the rest of membrane proteins cannot be released in these conditions, as they are tightly associated with the membrane, therefore being called integral membrane proteins (IMPs) (Alberts *et al.*, 2014).

Most integral proteins are embedded in membranes by hydrophobic α-helical sequences, of approximately 20 amino acids in length. This hydrophobic segment is called transmembrane domain (TMD), and it must be protected from the aqueous cytosol as soon as it emerges from the ribosome until it reaches the organelle, in which it will be inserted (Denic V., 2012).
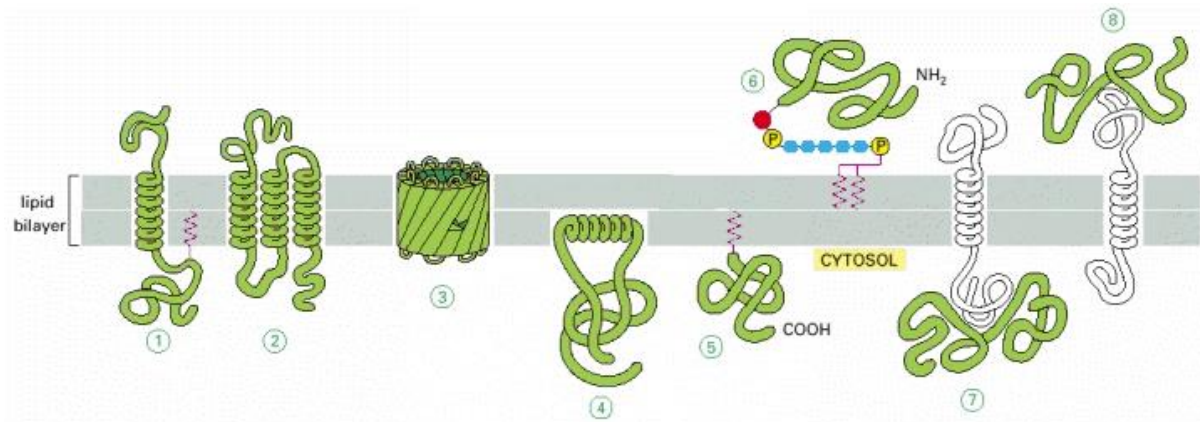
Cartoon 1: Membrane proteins and association with the lipid bilayer. Most trans-membrane proteins extend across the bilayer as (1) a single α-helix, (2) as multiple α-helices, or (3) as a β-barrel. Other membrane proteins are exposed at only one side of the membrane (4 and 5). Others are attached to the bilayer solely by a covalently attached lipid chain in the cytosolic monolayer (5), or via an oligosaccharide linker, to phosphatidylinositol in the non-cytosolic monolayer (6). Finally, many proteins are attached to the membrane only by non-covalent interactions with other membrane proteins (7,8). Reproduced from Alberts *et al.*, 2014.

IMPs that are destined to the plasma membrane or intracellular compartments of the secretory and endocytic pathways are initially inserted into the endoplasmic reticulum (ER). Here the fate of the protein is decided: if the final topology is correctly achieved the protein can proceed to its final location of function; if not, quality-control pathways would recognise it and send the protein for degradation (Shao and Hegde, 2011).

In order to be targeted for insertion into or translocation across membranes, proteins must have specific signals, which will be recognised by different machineries. Some examples of such signals include the secretory pathway signal sequences, mitochondrial targeting sequences and chloroplast leader peptides, each of which are recognised by well-characterised, dedicated protein machinery (Alberts *et al.*, 2014).

2.1.1 Insertion of membrane proteins into the ER: The Signal Recognition Particle (SRP)

The co-translational membrane insertion mediated by the cytosolic signal recognition particle (SRP), the ER-localised SRP receptor (SR) and the ER-localised translocon consisting of the Sec61 complex, is widely accepted as the most prominent pathway for delivery of IMPs and soluble proteins of the secretory pathway to the ER (Elvekrog and Walter, 2015). This co-translational molecular machinery is conserved across all domains of life, though it varies in composition and function. Since the discovery of the SRP in mammals more than 30 years ago, many groups of research have described in detail how this molecular machine works.

Both SRP and SR contain GTPase domains. The GTP-dependent association of SRP into a tight complex with the ribosome and the nascent chain is crucial for targeting the ribosome–nascent chain complex (RNC) to the protein translocation apparatus at the membrane. In eukaryotes, SRP is composed of six proteins and one 7S RNA molecule (Elvekrog and Walter, 2015).

As the protein is synthesized at the ribosome, this pathway begins when a hydrophobic segment of the protein, typically an N-terminal signal sequence (destined for subsequent removal) or a TMD close to the N-terminal, emerges from the ribosomal exit tunnel. This hydrophobic segment is then recognised and bound by the SRP (Mayerhofer P., 2016). The conserved protein of SRP, called SRP54, is responsible for signal sequence recognition and RNC targeting to the membrane. SRP54 contains three domains, termed the N, G and M domains. The C terminal, methionine-rich M domain, contains the signal-sequence-binding site and provides the primary contact to SRP RNA. It is connected through a flexible linker to the N-terminal four-helix bundle and GTPase domains (NG domain), responsible for the initial binding of SRP to the large subunit of the ribosome. (Elvekrog and Walter, 2015).

This interaction occurs at the ribosomal exit tunnel and persists until the transfer to the receptor at the ER, which guarantees a constant shielding of the TMD during targeting. Furthermore, the interaction of the SRP with the TMD transiently slows translation, which increases the kinetic window for targeting. At the ER membrane, the SRP-RNC complex

interacts with the SRP receptor in its GTP-bound state. SRP and its receptor mutually stimulate each other's GTPase activity, with the consequent delivery of the RNC to the translocon (Elvekrog and Walter, 2015).

At the ER membrane, the translocon is centrally composed of the Sec61 complex. The X-ray crystal structure of the archael complex revealed Sec61 is a conserved heterotrimer composed of α, β, and γ subunits (van der Berg *et al.*, 2004). Sec61α is the component that forms the translocation channel; the channel can also open laterally towards the lipid bilayer providing a lateral path for TMD insertion into the membrane. The lateral opening of the channel was compared to the opening of a clam shell, dependent on a hinge region opposite to the part of the structure that opens. In the absence of a signal sequence, the lateral gate is closed and also the channel from cytosol to ER lumen is plugged by a short α-helix (called the plug). It was suggested that the bound signal sequence causes the opening of the pore channel by displacement of the plug. Subsequently, the work of Voorhees *et al.* (2014), who resolved the structure of mammalian Sec61 complex by cryo-EM, was important to better understand the mechanistic basis of translocation across the ER membrane. They provided a possible model for the exchange of an RNC from SRP to a translocon: a first stage of activation involves binding of the ribosome, which primes the Sec61 channel by opening of the lateral gate. The lateral gate is an exit portal, which is opened for the entry and the insertion of TMDs of membrane proteins by breaking hydrophobic interactions and hydrogen bonds; then, in the second stage of activation, a suitable substrate can exploit the primed Sec61 by binding to and further opening the lateral gate. The opening of the lateral gate in the first step is also important for decreasing the energetic barrier necessary for translocation.

Presumably, when the nascent chain is transferred from SRP to Sec61, the SRP-SR complex is disassembled, and the translational pause is relieved. These events are coordinated by the GTPase activities of SRP54, and the α and β subunits of SR (Shao and Hegde, 2011). The principal steps in protein targeting to the ER are summarised in Cartoon 2.

Besides the Sec61 complex, several accessory factors have been proposed to assist the biogenesis of membrane proteins. Some of these factors are enzymes (such as signal peptidase complex , which removes signal peptides from nascent proteins as they are translocated into the ER, and oligosaccharyl-transferase complex, which transfers a 14-sugar-oligosaccharide to nascent proteins), putative accessory factors [such as the translocon associated protein (TRAP) complex and translocating-chain associating membrane (TRAM) protein], membrane chaperones (such as Calnexin and Bip), and other proteins, such as ribosome-associated membrane protein 4 (RAMP4), p180, Sec62, Sec63. In yeast, the Sec62-Sec63 complex mediates post-translational insertion of some secretory proteins into the ER; the feature that determines post-translational translocation is the presence of a weak signal sequence, poorly recognised by SRP (Panzner *et al.*, 1995). Also in mammals the depletion of Sec62 or Sec63 leads to defects in translocation of a subset of secretory proteins (Lakkaraju *et al.*, 2012; Lang *et al.*, 2012). Sec63 appears to play a substrate-specific role in the initial insertion of certain precursors into the complex, i.e., it helps in the opening of the Sec61 complex when the signal peptides are not strong enough to do it. More recently, *Jung et al.* (2014) have shown that mutations in the N-terminal domain of Sec62 disrupt the interaction with Sec63 and lead to defects in translocation of moderately hydrophobic TMDs of single- as well as multi-spanning membrane proteins.

The Sec62/63 complex is involved in the post-translational translocation of soluble precursors. The vast majority of membrane proteins, instead, is inserted by the co-translational pathway. Nevertheless, a subpopulation of membrane proteins that inserts post-translationally does exist. Post-translationally inserted membrane proteins include those that contain TMDs that are not exposed to the cytosol long enough during translation for efficient recognition by ribosome-bound SRP. Translational termination of such proteins makes them poor SRP substrates, necessitating their recognition, targeting, and insertion by a purely post-translational mechanism. Examples of such proteins include extremely small membrane proteins of either orientation and tail anchored (TA) proteins that contain their only TMD within ~40 residues of the C terminus (Borgese and Fasana, 2011; Borgese N., 2015).
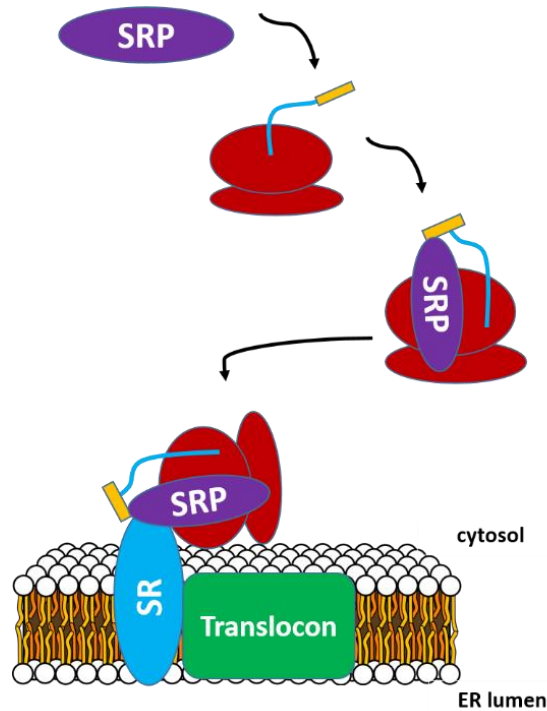
Cartoon 2: The SRP-mediated co-translational protein targeting. A nascent polypeptide with a signal peptide (in orange) emerges from the ribosome (in red) and is recognised by the SRP. The RNC–SRP complex is then targeted to the membrane through interactions between the SRP and its SR.

2.1.2 Insertion of membrane proteins into mitochondrial membranes

2.1.2.1 The TOM/TIM complexes:

The vast majority of proteins destined for the mitochondria are encoded in the nucleus and synthesized in the cytoplasm. So, functionality and proliferation of mitochondria depends on the continuous influx of proteins through the general Translocase of the Outer Membrane (TOM complex). The TOM works in conjunction with the translocase of the inner membrane (TIM) to translocate proteins into the mitochondrion (Cartoon 3). The central pore-forming TOM subunit is the β-barrel protein Tom40. Furthermore, the TOM complex comprises three receptor proteins, Tom20, Tom22, and Tom70, and three small

Tom proteins, Tom5, Tom6, and Tom7, which play distinct roles in the assembly and stability of the complex (Bohnert *et al.*, 2015).

Assembly of the mature multi-pore TOM complex depends on the presence of Tom22. This central Tom receptor spans the outer membrane with a single α-helix and exposes soluble domains to the cytosol and the intermembrane space that both interact with incoming precursor proteins. In the absence of Tom22, Tom40 and small Tom proteins are found in small, single-pore complexes with residual import capacity. The peripheral Tom receptors Tom20 and Tom70 are composed of N-terminal α-helical transmembrane segments and C-terminal cytosolic domains that recognise targeting signals in the different classes of mitochondrial precursor proteins. Whereas Tom20 preferentially binds cleavable pre-sequences of inner membrane and matrix proteins, Tom70 recognises internal hydrophobic signals of polytopic membrane proteins (Bolender *et al.*, 2008).

After translocation through the TOM complex, the incoming precursors with N-terminal targeting sequences are handed over to the pre-sequence translocase of the inner membrane (TIM23). Tim50 and Tim21, two proteins in the intermembrane space, were found to participate in this transfer (Schulz *et al.*, 2011). Tim50 binds to the protein emerging at the *trans* side of the TOM channel and promotes binding of the pre-sequence to the intermembrane space domain of Tom22 (Cartoon 3). Tim21 connects TOM and TIM complexes by direct but transient binding to Tom22. Importantly, the membrane potential ($\Delta\psi$) is crucial to drive pre-protein translocation across the inner membrane: it activates the channel-forming protein Tim23 and exerts an electrophoretic effect on the positively charged pre-sequences (Bolender *et al.*, 2008).

Another complex of the inner membrane is the TIM22 complex, which is essential for the insertion of polytopic inner membrane precursor proteins. The pore-forming core subunit of this complex is Tim22, a hydrophobic protein which is itself a substrate of the carrier translocase (Rehling *et al.*, 2003). After Tim22, other subunits of TIM complex were discovered: Tim9, Tim10, Tim12, Tim18 and Tim54. As in TIM23, the import of proteins across or into the inner membrane via TIM22 requires $\Delta\psi$, which is generated by the respiratory chain.

2.1.2.2 Insertion of MOM proteins:

More relevant to the subject of my thesis is the mechanism of targeting/integration of MOM proteins. Multiple pathways have been identified, some of which involve the participation of the TOM complex and some of which do not.

The MIM machinery

The mitochondrial import (MIM) machinery plays a central role for the insertion of MOM proteins that are anchored by α-helical transmembrane segments. The MIM component Mim1 was originally identified as a protein involved in biogenesis of the TOM complex. Mim1 forms oligomeric complexes in the outer membrane, together with Mim2 that facilitate the insertion of signal-anchored proteins (N-terminal transmembrane segment), like Tom20 and Tom70, in a process not requiring the Tom40 import channel. In addition, the MIM complex cooperates with the Tom70 receptor to promote the biogenesis of Ugo1, a polytopic outer membrane protein involved in mitochondrial fusion. Future work will be needed to elucidate the molecular basis of precursor interaction with the MIM complex and the mechanism of transmembrane segment insertion (Bohnert *et al.*, 2015). It must be mentioned that TA proteins, whose TMD is close to the C-terminus, do not require the MIM complex for their insertion.
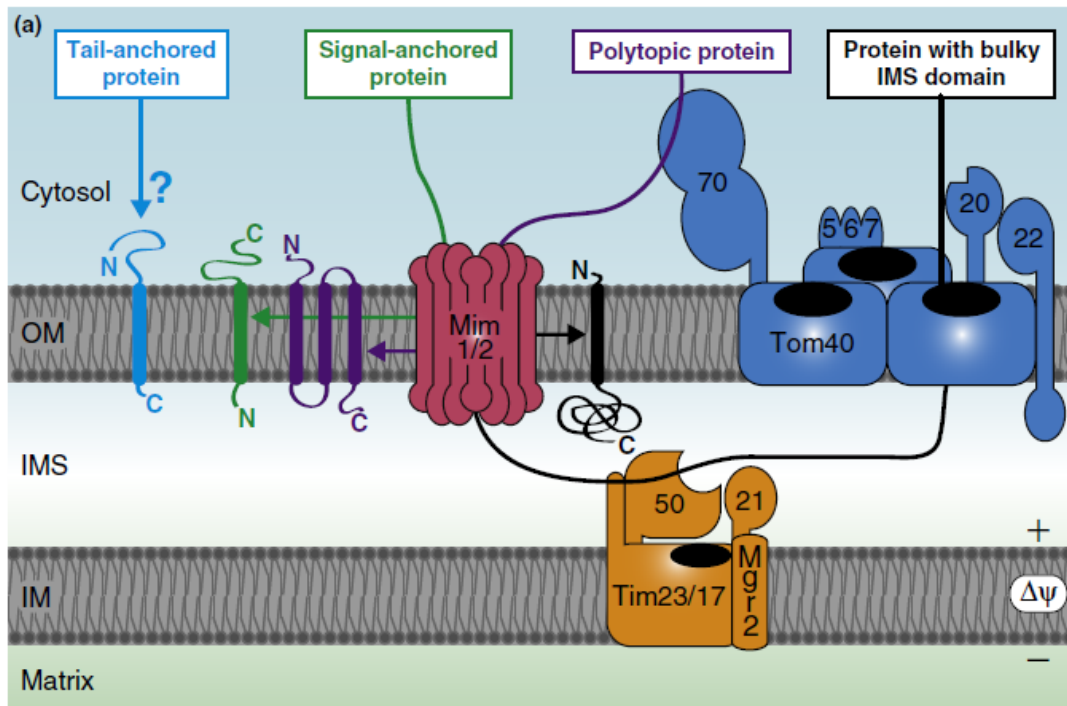
Cartoon 3: Outer membrane protein biogenesis. The import of MOM proteins with a single N-terminal α-helix (signal anchor) depends on the MIM machinery (pink). Polytopic α-helical proteins require MIM and Tom70, a receptor of the translocase of the outer membrane (TOM complex; blue). Om45, a MOM protein with a bulky intermembrane space domain, depends on TOM, TIM23 and MIM. Till date, a proteinaceous insertion machinery has not been identified for TA proteins. Reproduced from Bohnert *et al.*, 2015.

2.1.2.3 β-barrel proteins:

In eukaryotic cells, β-barrel membrane proteins are exclusively found in the outer membrane of endosymbiotic organelles like mitochondria, which derived from the outer membrane of Gram-negative bacteria. These proteins rely on a two-step import, with the transfer from the TOM complex to the sorting and assembly machinery - SAM (Ulrich *et al.*, 2014). The small TIM proteins help in this process, by guiding the hydrophobic β-barrel proteins through the aqueous intermembrane space to the SAM complex in the outer membrane (Cartoon 4). Here, SAM complex recognises β-barrel proteins by a β-signal in the C-terminal β-strand. This two-step mechanism is one evidence of the endosymbiotic

ancestry of mitochondria, since it is comparable to the β-barrel assembly machinery in bacteria (BAM). Indeed, Sam50, the central essential subunit of the SAM complex is a β-barrel protein itself and related to BamA of the BAM. TOM and SAM complexes directly associate to form a transient supercomplex in the outer membrane, where Tom22 plays a crucial role for the assembly and efficient transfer of β-barrel precursors. In the SAM complex Sam50 acts together with another essential subunit, Sam35, and two further subunits, Sam37 and Mdm10. (Ulrich *et al.*, 2014; Höhr *et al.*, 2015).
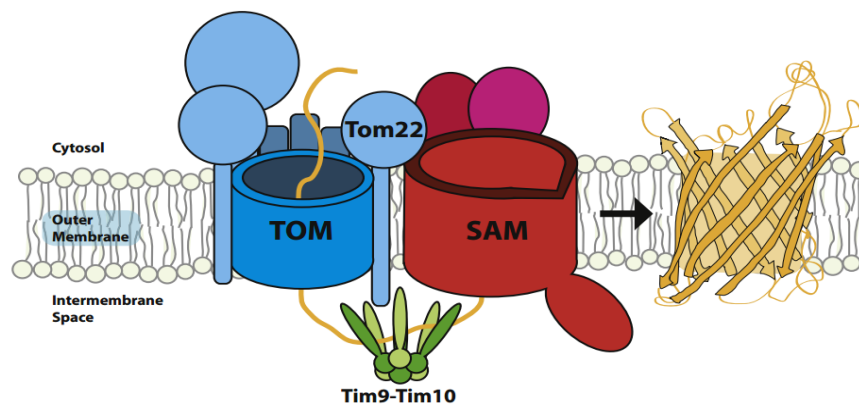
Cartoon 4: Import of β-barrel proteins. The TOM and SAM machineries form a supercomplex for β-barrel protein import. Reproduced from Höhr *et al.*, 2015.

2.1.3 Insertion of membrane proteins into peroxisomes

Peroxisome membrane proteins (PMPs) play essential roles in the biogenesis of this organelle and in co-ordinating peroxisomal metabolism with pathways in other subcellular compartments through transport of metabolites and the operation of redox shuttles (Theodoulu *et al.*, 2013). Early studies indicated that PMPs are synthesised on free polysomes in the cytosol and subsequently inserted post-translationally into the membranes

of pre-existing peroxisomes. This process requires both cytosolic factors and proteinaceous components at the peroxisomal membrane. Three peroxins, namely PEX3, PEX16 and PEX19, were identified to be essential for the post-translational targeting and insertion of PMPs into the peroxisomal bilayer in several species (Cartoon 5) (Mayerhofer P., 2016).

PEX19 is a predominantly cytosolic protein with a conserved C-terminal farnesylation site. This protein serves as a PMP chaperone, preventing aggregation and degradation of newly synthesized peroxisomal proteins (Theodoulou *et al.*, 2013). PEX19 has been shown to bind to a broad range of PMPs at their specific peroxisome targeting signal, termed mPTS. PMPs may contain single or multiple mPTSs, which are composed of a cluster of positively charged residues (sometimes mixed with hydrophobic residues) flanked by one or two TMDs (Mayerhofer P., 2016).

Algorithms have been developed for the *in silico* prediction of mPTSs, and based on mPTS content and mPTS properties, PMPs were initially classified into two groups: class 1 PMPs (harboring a mPTS1) rely on their interaction with PEX19 to be post-translationally imported into peroxisomes, whereas the mPTS2 of class 2 PMPs are PEX19-independent and traffic to peroxisomes via the ER. Initially strictly based on whether or not a mPTS binds to PEX19, PMPs were later sorted by new criteria, for instance ER passage or PEX16-dependence (Buentzel *et al.*, 2015; Theodoulou *et al.*, 2013).

Except for yeast TA PMPs, so far only PEX3 and PEX22 were identified to belong to class 2. Hence, all other known PMPs belong to class 1 and thus depend on PEX19-binding for their peroxisomal targeting. The way that this happens is the following: after the formation of PEX19·cargoPMP complexes in the cytosol, PEX19 docks on PEX3 at the peroxisomal membrane, thereby forming a trimeric PEX3·PEX19·cargoPMP complex.

Besides PEX19 and PEX3, a third peroxin plays a key role in early peroxisomal membrane genesis in mammals: PEX16. This integral PMP was reported to act as receptor for PEX3·PEX19 complexes at the peroxisomal membrane. However, this role of PEX16 is not conserved among species, and most yeasts, including *S. cerevisiae*, lack a PEX16 homolog (Mayerhofer, P. 2016).
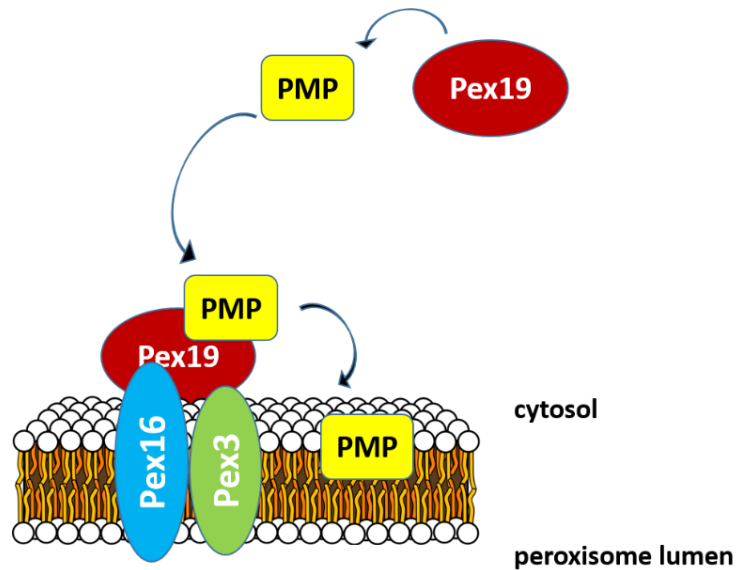
Cartoon 5: Post-translational insertion of PMPs. Pex19 binds to PMPs and transports them to the peroxisomal membrane, where it docks with a complex containing Pex16 and Pex3.

2.2 Tail-anchored proteins

The features and biogenetic pathways of TA proteins have been discussed in several reviews during the past years (Borgese *et al.*, 2003; Borgese *et al.*, 2007; Rabu *et al.*, 2009; Borgese and Fasana, 2011; Colombo and Fasana, 2011; Hegde and Keenan, 2011; Shao and Hegde, 2011; Chartron *et al.*, 2012; Denic V., 2012; Johnson *et al.*, 2013; Borgese N., 2015).

The defining feature of a TA protein is the presence of a single transmembrane domain (TMD) at the extreme C-terminus of the polypeptide. This TMD provides a targeting signal for the delivery of the protein to the correct subcellular compartment and also acts as an anchor that retains the polypeptide in the lipid bilayer once integration has taken place (Borgese *et al.*, 2007).

TA proteins are always oriented in the membrane of the organelle with the larger N-terminal region facing the cytosol (Cartoon 6). This part carries out the biological function of the polypeptide, as exemplified in the case of the SNARE proteins, in which the cytoplasmic domain plays a crucial role during vesicular transport.

When the ribosomes terminate the translation of TA proteins, the C-terminal TMD, which encodes the targeting information, is inside the ribosome tunnel, precluding the SRP-mediated co-translational targeting. Therefore, TA proteins take an alternative post-translational pathway for insertion into their target membranes - ER, MOM, chloroplast, peroxisomal, and prokaryotic cytoplasmic membranes. This biosynthetic route is what distinguishes TA proteins from classical type II membrane proteins (defined as proteins having a single TMD with N-cytosolic, C-exoplasmic orientation), which are delivered to the ER by the SRP-dependent co-translational pathway (Cartoon 6). By this biosynthetic criterion then - and considering that the last 40 amino acids of the nascent chain are sequestered within the eukaryotic large ribosomal subunit - TA proteins should be considered as such if the membrane-interacting domain is followed by no more than 30 residues.

Bioinformatic analyses indicate that TA proteins are well represented in all three domains of life (Borgese and Righi, 2010): over 50 are predicted to be expressed in yeast and in excess of 300 in humans. In eukaryotes, TA proteins can be found in the mitochondrial, peroxisomal, chloroplast outer membranes, and in intracellular compartments that are connected by the secretory and endocytic pathways, including the ER, Golgi, plasma membrane, endosomes and lysosomes. The ER acts as the entry point for the many different TA proteins that occupy the compartments of the secretory pathway. The ER is also involved in TA protein transport to peroxisomes by an indirect pathway that is prevalent in yeast. MOM and plastid TA proteins are instead targeted directly from the cytosol to their destination (Cartoon 7).

TA proteins play essential roles in basic cellular functions including vesicle trafficking, protein synthesis, protein quality control, organelle biogenesis and lipid metabolism (Table 1). Therefore, understanding the molecular mechanism of the biogenesis of TA proteins, in particular the post-translational membrane insertion of TA proteins, is an important issue in

cell biology, with implications for physiology and pharmacology as well. An example of how localisation and function of TA proteins could be modulated is offered by the anti-apoptotic protein Bcl-2. Bcl-2 is associated both with the ER and MOM, where it exerts its biological activity. Studies with forms of Bcl-2 engineered to localise exclusively to the ER or to the MOM have shown that in the two localisations it has different functions: the ER associated form has been implicated in the maintenance of calcium homeostasis and, although protective against a limited subset of apoptogenic signals, may in some cases be more effective than the MOM targeted protein. Thus, cell's response to death and survival could be controlled by modulation of the targeting of Bcl-2 (Schinzel *et al.*, 2004; Borgese *et al.*, 2003).

Table 1: Examples of TA proteins and their functions (adapted from Borgese and Fasana, 2011).

Target membrane	TA protein	Function
ER	Sec61b	Protein translocation
	Ramp4	Protein translocation
	Synaptobrevin-2	SNARE
	Cytochrome b5	Lipid metabolism in ER
	Bcl-2	Regulation of apoptosis
MOM	Small TOM proteins	Protein translocation
	Cytochrome b5 (mitochondrial isoform)	Enzymatic function
	VAMP-1B	SNARE
	Bcl-2	Regulation of apoptosis
	Fis1	Mitochondrial fission
Peroxisomes	Pex26	Peroxisome biogenesis
	Fis1	Peroxisomal fission
Chloroplast Outer Envelope	Bcl-XL	Regulation of apoptosis
	Bak	Regulation of apoptosis
	At1g26340 (cytb5 isoform)	Enzymatic function
	Toc33,34	Protein translocation

Because of the small number of polar residues downstream to the hydrophobic domain, it was initially difficult to verify the exact topology of TA proteins. This problem was overcome by the use of TA proteins with N-glycosylation sites engineered to the extreme COOH-terminal polar region. Since glycosylation takes place in the ER lumen *in vivo* as well in ER-derived microsomes *in vitro*, when such modification occurs it means that TA proteins can translocate their C-terminus across the lipid bilayer; plus, when a recombinant or *in vitro* synthesised TA protein with a N-glycosylation site is used, glycosylation can demonstrate that the translocation occurs post-translationally. Unfortunately, this technique cannot be applied to the other target membranes of TA proteins (e.g. the MOM), in which the transmembrane topology of the C-terminal TMD has not been unequivocally demonstrated yet.
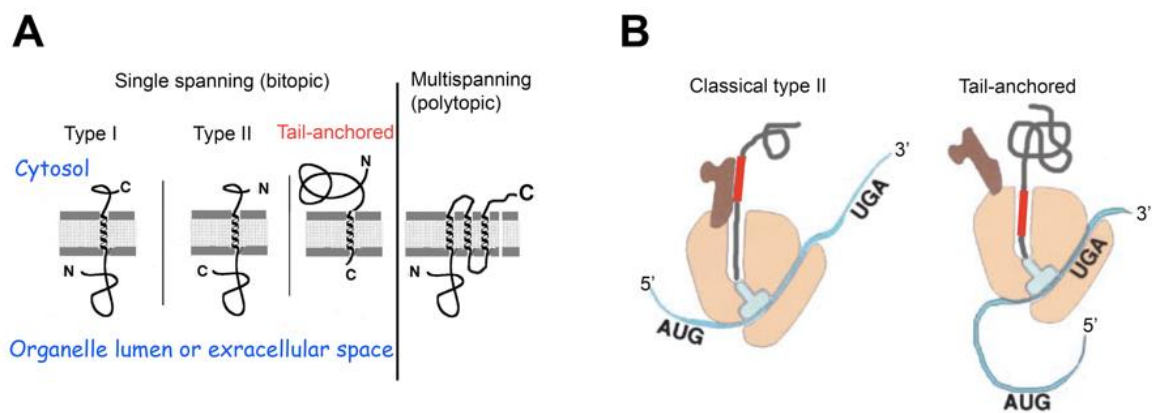
Cartoon 6: Topology of TA proteins. Panel A compares the topology of TA proteins with that of other single-spanning and of polytopic transmembrane proteins. Panel B shows how the tail anchor (red) of TA proteins (right), unlike the signal anchor (red) of classical type II proteins (left), is unable to interact co-translationally with SRP (brown). Reproduced from Borgese N. (2015).
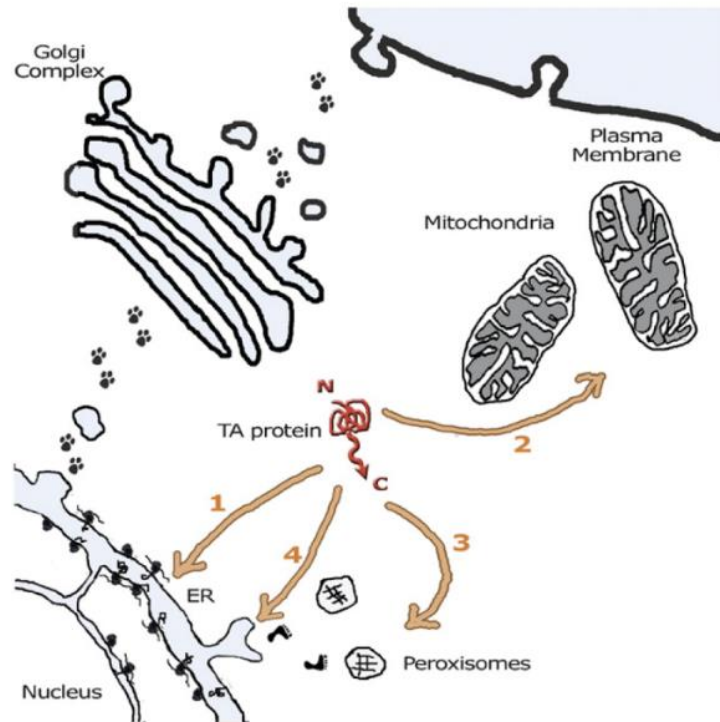
Cartoon 7: Biogenetic pathways of TA proteins. After release from ribosomes, a TA protein (red) can insert into the ER (1), the MOM (2) or the peroxisomal membrane (3) (and into the chloroplast outer envelope in plant cells, not depicted here). TA proteins may also reach the peroxisomal membrane by targeting first to specialised ER subdomains (4), followed by maturation of these domains into the mature organelle (foot prints). Once targeted to the ER, TA proteins can then be transported through the secretory pathway (paw marks). Reproduced from Borgese *et al.*, 2007.

2.2.1 Targeting of tail-anchored proteins to the ER

TA proteins are prone to aggregate in the cytosol due to the hydrophobicity of their C-terminal TMD. Therefore, while in transit to the target membrane, there needs to be a mechanism by which newly synthesized TA proteins are protected from aggregation. Although there is some data to suggest that some TA proteins with a less hydrophobic TMD,

such as cytochrome b5, could integrate into membranes without the assistance of specialised machinery (Brambillasca *et al.*, 2005, 2006; Colombo *et al.*, 2009), most studies suggest that the biogenesis of TA at the ER involves one or more cytosolic factors. To date, four different cytosol-dependent mechanisms have been described for secretory pathway TA proteins: Hsp40/Hsc70-mediated (Rabu *et al.*, 2008), SRP-mediated (Rabu *et al.*, 2009), Get3/TRC40-mediated (Stefanovic and Hegde, 2007; Schuldiner *et al.*, 2008; Favaloro *et al.*, 2010) and SND-mediated (Aviram *et al.*, 2016). Of these, the Get3/TRC40 pathway plays the most important role and is by far the most well-characterised.

2.2.1.1 The Get3/TRC40 pathway

As mentioned before, in the co-translational pathway, SRP serves both a chaperoning and a targeting role, ensuring complete shielding of its hydrophobic cargo during cytosolic transit until the translocon is reached. Similarly, the hydrophobic TMD of TA proteins must be shielded from aggregation in the cytosol. The search for such a shielding/targeting factor led to the identification of a highly conserved, cytosolic P-loop ATPase that was named TRC40 for TMD recognition complex subunit of 40 kDa (Stefanovic and Hegde 2007; Favaloro *et al.*, 20010). TRC40 is evolutionarily related to the bacterial arsenite transport factor ArsA (hence its original annotation as Asna-1, for arsenical pump-driving ATPase protein) and has emerged as a mammalian chaperone that maintains TA proteins in soluble state and brought the breakthrough that led to understanding the molecular mechanism of the post-translational membrane insertion of TA proteins.

In parallel with these findings, a comprehensive genetic interaction study of the early secretory pathway in yeasts had identified a novel protein complex involved in Golgi to ER traffic, termed the Golgi-to-ER Traffic (GET) complex. The GET complex is composed of Get1 and Get2, both of which are membrane proteins, and Get3, a cytosolic ATPase (Schuldiner *et al.*, 2008). Importantly, Get3 is a yeast homolog of mammalian TRC40 and it was discovered that its role in ER/Golgi traffic was an indirect effect, due to mistargeting of essential SNARE proteins in its absence. Hence, the meaning of the acronym GET was

changed to ''Guided Entry of TA Proteins'', and the yeast GET complex provided a basic framework for delineating the molecular mechanism of the post-translational membrane insertion pathway. The mammalian TRC40 pathway is less well characterised than the yeast Get3 system, however, with the exception of Get2, homologues of all the *S. cerevisiae* components are expressed in higher eukaryotes and are thought to carry out similar functions (Denic V., 2012). Below, both systems will be described in detail:

- **The GET pathway in yeast:**

In the GET pathway, Get3 selectively chaperones the TMDs of newly synthesized TA proteins in the cytosol and targets them to the ER membrane, where Get1 and Get2 constitute a receptor complex for Get3 and drive insertion of the TA proteins into the ER membrane (Schuldiner *et al.*, 2008). The sum of biochemical and structural evidence has revealed a surprisingly elaborate pre-targeting mechanism that facilitates TMD recognition by Get 3.

An *in vitro* reconstitution study of the GET complex demonstrated that Get1, Get2 and Get3 are sufficient for mediating the post-translational membrane insertion of TA proteins (Schuldiner *et al.*, 2008). However, in addition to these three core components, the yeast GET pathway has been shown to employ three additional factors, Stg2 (small, glutamine-rich, tetratricopeptide repeat protein 2), Get4 and Get5. Entry of newly synthesized TA proteins into the GET pathway begins with efficient capture by Sgt2, through binding of its C-terminal region to the TMDs of TA proteins soon after release from the ribosomes. This chaperone shields TMDs after they are released from the ribosome to prevent TA protein aggregation in the cytosol or mistargeting to mitochondria. Sgt2 interacts with the Get4-Get5 complex through the ubiquitin-like domain of Get5, serving as a platform for escorting newly synthesized TA proteins to Get3 (Chartron *et al.*, 2010; Denic V., 2012).

The structural and biochemical studies of Get3 indicate that it is a homodimeric ATPase that switches between open and closed conformations depending on the nucleotide loading, the TA protein loading and the binding proteins (Mateja *et al.*, 2009). In the ATP-bound state, Get3 adopts the closed conformation favourable for binding to the TMDs of TA

proteins. The binding of the Get4-Get5 complex to ATP-bound Get3 inhibits the ATPase activity of Get3, thereby priming Get3 for capturing the TA proteins (Gristick *et al.*, 2014). Upon receiving the TA proteins from Sgt2, Get3 activates the ATPase activity but delays ADP release, leading to formation of the ADP-bound Get3-TA protein complex competent for ER targeting.

The integral membrane proteins Get1 and Get2 form the ER membrane receptor complex required for TA-insertion by Get3. The flexible cytoplasmic domain of Get2 initially captures the Get3/TA-protein complex. As Get3 approaches the membrane, Get1 displaces Get2 and then facilitates a transition from closed to open Get3 (Mateja *et al.*, 2009; Stefer *et al.*, 2011). Indeed, the coiled-coil region of Get1 inserts like a wedge into the TA binding groove of Get3, forcing it into the open conformation with the consequent release of the TA to the bilayer. It has also been observed that the TMDs of Get1 and Get2 interact with the TMD of the TA protein near the cytosolic side of the ER membrane. These transmembrane interactions facilitate release of the TA protein from the Get3 and mediate the subsequent entry of the TA protein into the ER membrane. Therefore, it has been suggested the TMDs of Get1 and Get2 are not simple membrane anchors for their cytoplasmic domains but constitute a membrane insertase for TA proteins (Wang *et al.*, 2014).

Importantly, whereas mutations in components of the GET system are lethal in higher eukaryotes, yeast cells survive in their absence. Nevertheless, deletion of the GET3 gene in yeast leads to several, seemingly unrelated phenotypes, including hygromycin sensitivity, copper and H_2O_2 sensitivity, heat sensitivity and the inability to grow on iron-limiting media (Schuldiner *et al.*, 2008). Recently, a study by Voth *et al.* (2014) showed that upon exposure to ATP-depleting oxidative stress conditions, Get3 undergoes major conformational rearrangements, which appear to bury the proposed TA-binding site on Get3 and turn Get3 into an ATP-independent chaperone. They have demonstrated that oxidized Get3 binds unfolded proteins and prevents their irreversible aggregation, protecting cells against oxidative stress, and that, upon restoration of normal cellular ATP levels, Get3 returns into its initial dimer structure and presumably releases its substrate for refolding or degradation.

- **The TRC40 pathway in mammalian cells:**

Compared to the GET system, the TRC40 pathway employs another factor specific to mammals. Indeed, the pre-targeting complex, in addition to the mammalian homologues of Get4 and Get5 (TRC35 and Ubl4A, respectively), contains a third protein, called Bag6. Bag6 interacts both with Ubl4A and TRC35, presumably acting as a bridge between them, as the Get4/5 homologues lack the features of the yeast proteins required for direct interaction (Chartron *et al.*, 2012).

Like in yeast, the newly synthesised TA substrate is first captured by the mammalian Sgt2 homologue, known as SGTA, and then handed over to TRC40 via the pre-initiation complex, known as the Bag 6 complex. The Bag6 complex not only has the ability to load TA proteins onto TRC40, but also mediates protein degradation pathways for mislocalised membrane proteins, retro-translocated ER proteins and other defective proteins. When the TMD of a TA protein escapes TRC40 binding, the Bag6 complex captures the TMD to prevent undesired aggregation in the cytosol, leading to ubiquitination of the mislocalised membrane protein followed by proteasomal degradation.
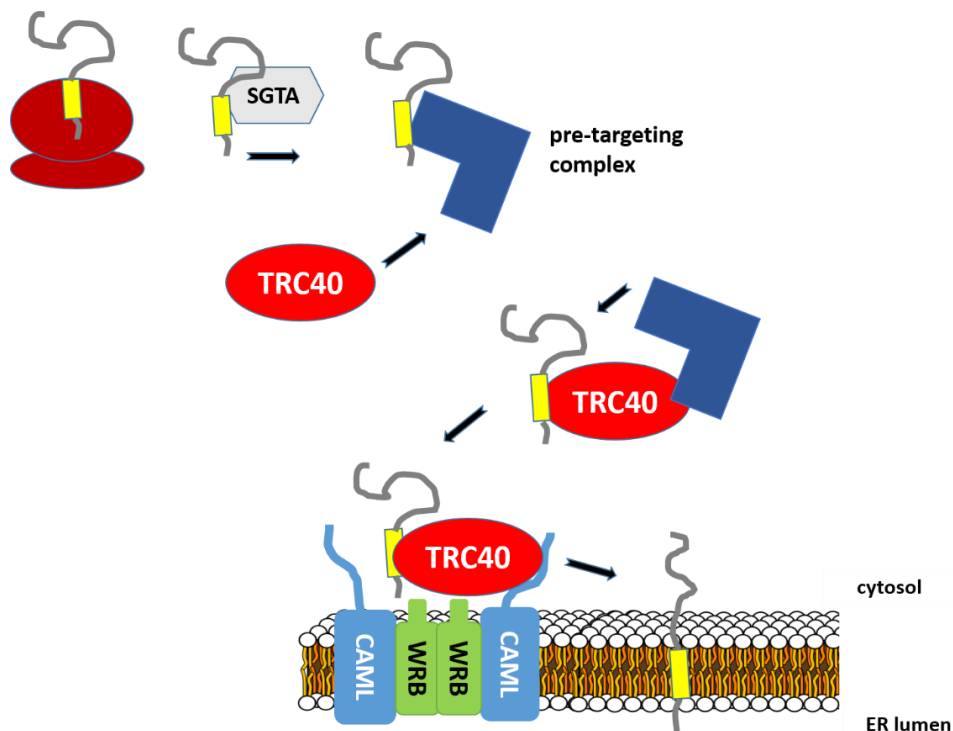
Cartoon 8: The TRC40 pathway. The pre-targeting complex composed of Bag6, TRC35 and UBL4A (depicted in dark blue) captures the TMD (in yellow) of the TA protein soon after release from the ribosome, facilitating loading of the TA protein onto TRC40. CAML binds to TRC40, recruiting the TRC40-TA protein complex to the ER membrane. CAML and WRB constitute the ER membrane receptor complex for the TRC40-TA protein complex, driving post-translational insertion of TA proteins into the ER membrane.

SGTA has emerged as a key regulator in macromolecular quality control, in addition to its established roles in the biogenesis of TA membrane proteins and proposed roles in hormone receptor signalling. Human SGTA is a 34 kDa protein made up of 313 amino acids that assembles as a homodimer, with each chain comprising three structural domains: a N-terminal dimerization domain, a central tetratricopeptide repeat (TPR) domain, and a C-terminal domain which includes a 39 amino acid glutamine rich region (Wunderley *et al.*, 2014; Roberts *et al.*, 2015). SGTA shields TA proteins from the aqueous cytosol, by recognising and binding to the hydrophobic exposed regions, preventing them from misfolding and aggregation. Then, the subsequent fate of the proteins is determined: the protein can be refolded to their native conformation, targeted to the correct destination or processed for degradation. Since it was demonstrated that SGTA directly binds to Bag6 and the TMDs of TA proteins in the cytosol, most likely TA proteins could be passed from SGTA to the Bag6 complex. Furthermore, a recent structure-based study of the Bag6 complex suggests that SGTA can also directly hand off TA proteins to TRC40. Thus, the sorting step of TA proteins by the Bag6 complex in mammalian cells is more complex than in yeast (Roberts *et al.*, 2015).

As said before, TRC40 is a highly conserved ATPase and the mammalian homolog of yeast Get3. The mammalian ER receptor for TRC40 is now generally believed to be constituted by the WRB/CAML complex. Mammalian WRB (tryptophan-rich basic protein), which shows sequence similarity to yeast Get1, is predicted to have three TMDs and a cytosolic coiled-coil domain. WRB binds to TRC40 through the cytoplasmic coiled-coil domain, mediating the post-translational insertion of TA proteins into the ER membrane in mammalian cells (Vilardi *et al.*, 2011).
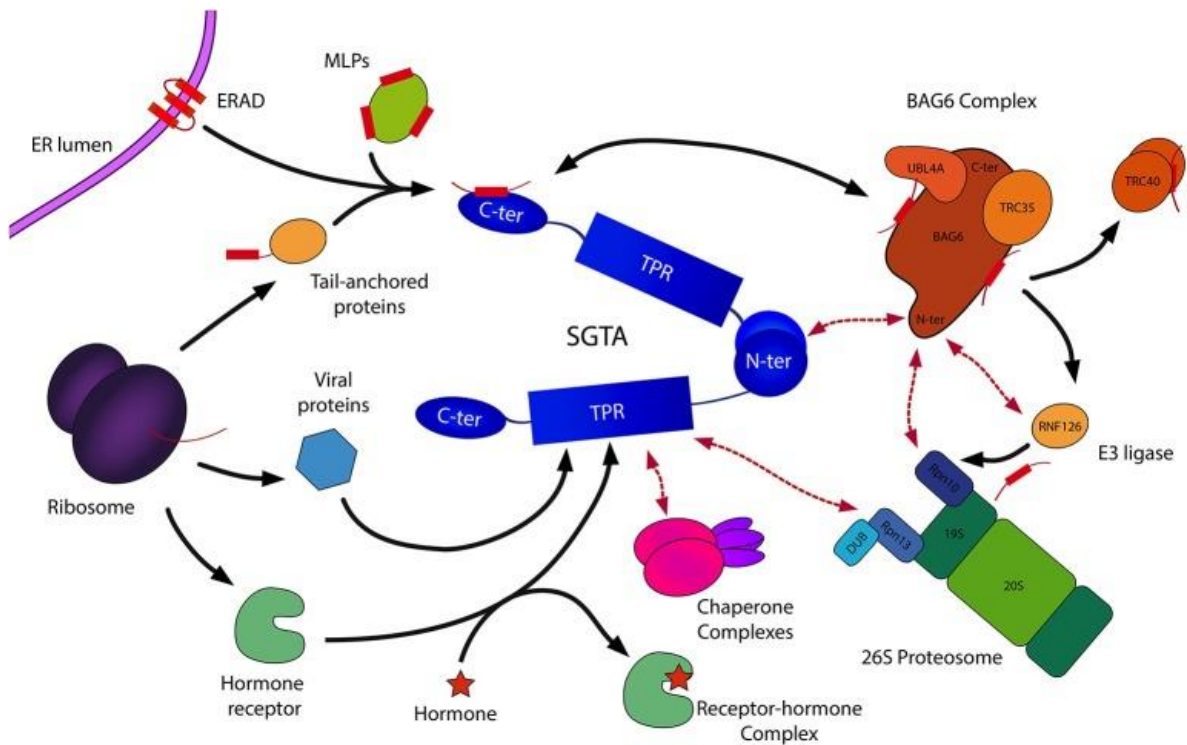
Cartoon 9: Biological roles of SGTA. SGTA is involved in the quality control of hydrophobic substrates, a process mediated by its C-terminal domain in collaboration with the Bag6 complex. The Bag6 complex interacts with SGTA via its ubiquitin-like domains (UBLs). Hydrophobic substrates bound to the Bag6 complex are ubiquitinated by the actions of the E3 ligase RNF126, thus targeted for proteasomal degradation. Additionally, SGTA's role extends to the shielding of exposed hydrophobic regions on TA proteins facilitating their post-translational integration into the ER. This enables the handover of TA proteins to the downstream TRC40 targeting complex. Furthermore, SGTA has been implicated in hormone receptor signalling, and has been associated with viral lifecycles. SGTA's interactions with Hsp70/Hsp90 chaperones via its TPR domain may provide substrate access to additional branches of the global cellular quality control network. Reproduced from Roberts *et al.*, 2015.

WRB is also known as congenital heart disease 5 (CHD5) protein, because the human gene encoding WRB is located in the Down syndrome critical region of chromosome 21 (21q22.3) within the congenital heart disease region. WRB has been shown to regulate heart development in medaka fish. Although it has been shown to be 1.5 fold overexpressed in fetal fibroblasts of Down syndrome subjects (Colombo *et al.*, 2016), the link of WRB to congenital heart disease frequently associated with Down syndrome, remains unclear.

It was postulated that the TRC40 pathway will employ an ER membrane protein analogous to Get2. However, computer-based sequence similarity searches had failed to identify the mammalian gene homologous to yeast Get2. Yet, pulldown experiments with TRC40 as bait identified CAML (calcium modulating cyclophilin ligand) as a TRC40- and WRB-interacting protein (Yamamoto and Sakisaka, 2015). CAML binds to TRC40 through the N-terminal cytoplasmic domain and to WRB through the C-terminal TMDs. The CAML/WRB complex is sufficient to rescue TA protein insertion in ΔGet1/Get2 yeast cells (Vilardi *et al.*, 2011) and TA protein insertion competence is obtained by reconstituting the two proteins into liposomes (Colombo *et al.*, 2016). Thus, although CAML and Get2 are not homologous, the WRB/CAML complex constitutes an ER membrane receptor for the TRC40-TA protein complex, and CAML represents the functional homologue of Get2 (Yamamoto and Sakisaka, 2015).

A very recent study carried out by Colombo *et al.* (2016) revealed unanticipated complexities in the function and regulation of the TRC40 receptor. They found that a stoichiometric excess of one subunit over the other does not affect TA protein insertion and, in agreement, that endogenous CAML is present in a 5-fold molar excess over endogenous WRB both in rat liver microsomes and in cultured cells. Notwithstanding this stoichiometric imbalance, the levels of each of the TRC40 receptor subunits depend critically on the presence of the other one, since the knockdown of each receptor subunit decreases the levels of the other one. Interestingly, in mammals the deletion of TRC40 is embryonic lethal, suggesting that, unless TRC40 has some other yet unknown function, at least one essential TA protein critically depends on TRC40 for its targeting/insertion (Borgese and Fasana, 2011).

▪ The case of short secretory proteins:

Another issue that has been discussed recently is the connection between TRC40 pathway and ER targeting of secretory peptides. Because of their short length (<100 amino acids), these proteins may be released from the ribosome before SRP can capture them, but they still depend on Sec61 for translocation into the ER lumen. Surprisingly, TRC40 can recognise secretory peptides *in vitro* and promote their ER translocation by a WRB-dependent mechanism. These findings raise the intriguing possibility that the GET pathway can feed certain substrates to the Sec61 channel. Specifically, the translocation of preprocecropin A (ppcecA) into ER derived microsomes is inhibited by lanthanum ions, and the small molecule inhibitor eeyarestatin I (ES I), both of which perturb Sec61 mediated translocation (Cross *et al.*, 2009; Johnson *et al.*, 2013).

2.2.1.2 The Hsp40-Hsc70 mediated pathway

The constitutively expressed and stress-inducible 70-kDa heat shock proteins Hsc70 and Hsp70, respectively, are ubiquitous molecular chaperones that bind and release polypeptides in an ATP-dependent cycle. Hsp70 function is regulated by and often dependent upon Hsp40 co-chaperones, which are defined by their homology to the DnaJ chaperone in *Escherichia coli*. Hsp40s stimulate the ATPase activity of Hsp70s and, thus, stabilise the Hsp70-peptide complex (Wright *et al.*, 2008). Using an assay for ATP hydrolysis, a study by Fewell *et al.* (2004) identified and screened small molecules for their effects on endogenous and Hsp40-stimulated Hsp70 ATPase activity. The most important compound found was MAL3-101. A series of derivates with improved physicochemical attributes, including reduced molecular weight, increased solubility, and decreased lipophilicity were then synthesized. These included MAL2-11B, DMT002220 and DMT3024. On the other hand, MAL3-51 was found as a structurally related compound with little effect on chaperone function (Fewell *et al.*, 2004; Wright *et al.*, 2008). These inhibitors have been used to show essential roles for Hsc/Hsp70 in maintaining breast cancer and multiple myeloma cell

survival but have been also shown to effectively inhibit the post-translational translocation of the yeast secretory protein, prepro-α-factor, across the ER membrane *in vitro* (Rabu *et al.*, 2008).

The effect of the MAL-based inhibitors was tested also on TA proteins insertion into ER-derived microsomes and found to inhibit the integration of a subset of TA proteins (Rabu *et al.*, 2008). This subset of proteins is characterised by TMDs of comparatively low hydrophobicity. It was therefore suggested that when multiple options are available, proteins with particularly hydrophobic TA regions such as synaptobrevin and Sec61β appear to favour TRC40 or other pathways, whilst those with a less hydrophobic TA region, such as cytochrome b5, use Hsc-70 mediated and/or unassisted pathways (Johnson *et al.*, 2013).

Presumably, the role of the molecular chaperones during post-translational targeting is mainly to prevent the aggregation of hydrophobic regions of polypeptide (such as tail-anchor segments), thereby maintaining these substrates in a membrane-integration competent conformation (Rabu *et al.*, 2009). Instead, a specific targeting function of these chaperones is difficult to envisage, as an ER receptor for Hsc70 has not been identified so far.

2.2.1.3 The SND-mediated pathway

Most secretory and membrane proteins are targeted to the translocon via SRP and its ER membrane-resident receptor, SR. However, a protein whose targeting element is a low-hydrophobicity signal sequence (SS) or a TMD outside of the most N' window, as it is the case of TA proteins, may be overlooked by the SRP, and would require dedicated SRP-independent pathways for ER relay (Ast *et al.*, 2013; Aviram *et al.*, 2016). Indeed, a study from the group of Maya Schuldiner revealed that an unexpectedly large proportion of the ER-directed proteome, reaches the ER by SRP-independent pathways (Ast *et al.*, 2013). Recently, one SRP-independent targeting system was discovered in yeast and termed SND system (Aviram *et al.*, 2016). Through a systematic high-content screen in yeast, three uncharacterised proteins needed for correct targeting of SRP-independent substrates were discovered. These proteins were named SND (Srp-iNDependent): SND1 is predicted to be a

peripheral ribosome binding protein, and, in agreement, its GFP-fusion protein localises to the cytosol; SND2 is predicted to have four transmembrane domains and was shown to affect vacuolar Carboxypeptidase Y (CPY) processing. Interestingly, CPY is a known SRP-independent substrate; the third protein, SND3, was shown to affect the biogenesis of yeast acid phosphatase, another SRP-independent substrate, and was shown to localise to the ER. So, analogous to the SRP-dependent pathway, this novel pathway involves a cytosolic component (SND1), which binds to ribosomes and, subsequently, to its heteromeric receptor in the ER membrane (comprising SND2 and SND3) to achieve co- or post-translational delivery of precursor polypeptides to the Sec61 complex (Aviram *et al.*, 2016).

So far, a mammalian ortholog has been described only for SND2 (hSND2), which may or may not have the same function as its yeast ortholog and may be involved in TA protein targeting. Indeed, the SND proteins may have higher affinity for proteins with more central TMDs, but it seems that the three pathways, SRP, SND and GET, work in parallel to facilitate the ER targeting of proteins bearing TMDs in all possible relative positions.

2.2.1.4 The SRP-mediated pathway

Although SRP was thought to be involved exclusively in co-translational targeting, a possible unusual role of this ribonucleoprotein in post-translational targeting of TA proteins was suggested by work from the group of Stephen High. An *in vitro* crosslinking approach showed that the tail-anchor region of synaptobrevin 2 (Syb2) is transiently associated with the signal sequence-binding domain of SRP. Furthermore, the efficient membrane insertion of Syb2 requires an intact SRP receptor, which is consistent with the idea that SRP has a post-translational role in the binding and delivery of TA proteins to the ER membrane (Abell *et al.*, 2004; Rabu *et al*, 2009). This SRP-dependent mechanism was thought to favour a small subset of TA protein precursors with more hydrophobic TMDs. The role of SRP in TA protein targeting remains, however, controversial, because a previous study demonstrated SR-independent insertion of Syb2 into ER-derived proteoliposomes (Kutay *et al.*, 1995), and because the energy source for TA proteins insertion is provided by ATP hydrolysis, while SRP requires GTP.

2.2.2 Targeting of tail-anchored proteins to other organelles

An important and only partially resolved problem is the mechanism whereby TA proteins escape from the GET/TRC pathway to be delivered to other organelles. Get3/TRC40, as well as Sgt2/SGTA have broad specificity, in that a hydrophobic stretch of sufficient length close to the C-terminus is the only requirement to qualify as substrate. Thus, TA proteins destined to organelles other than the ER must have features that exclude them from the GET/TRC pathway. In general, a combination of moderate hydrophobicity of the TMD and the presence of positive charges in the tail region appears to serve this function, at least in mammals (Borgese N., 2015).

2.2.2.1 Mitochondria

TA protein delivery to the mitochondrial outer membrane (MOM) appears to depend on physico-chemical features of the tails, as mentioned in the preceding paragraph, and not on defined signals: these proteins are generally characterised by TMDs of moderate hydrophobicity, flanked on one or both sides by positive charges. In fungi and plants, the presence of flanking positive charges does not distinguish MOM from ER-targeted TA proteins, and moderate hydrophobicity seems to be the only clear hallmark for MOM targeting. An additional TMD feature reported to favour mitochondrial targeting is the propensity for α-helix formation (reviewed in Borgese and Fasana, 2011). These features would determine escape from the GET pathway and delivery to mitochondria (Hwang *et al.*, 2004).

It is currently unclear how the specific targeting of TA proteins to the MOM is achieved, since mitochondrial TA proteins do not utilise the general import machinery of the mitochondria. Although the TOM complex or Mim1 appear to be required for the insertion of certain MOM TA proteins (for example, Tom20 has been implicated in the import of Bcl-2), *in vitro* studies carried out with four TA substrates (Bak, BclXL and OMP25 in mammals and Fis1 in yeast) have excluded the participation of the TOM complex (Kemper *et al.*, 2008;

Setoguchi *et al.*, 2006). In these works, the C-terminal TMD is sufficient for MOM targeting and none of the import components at the outer membrane is involved in the insertion. In addition, to date, no cytosolic components have been identified for the targeting of TA proteins to the MOM. Interestingly, the unique lipid composition of the MOM, in particular the low content of sterols, appears to be important for the targeting, at least in yeast (Kemper *et al*, 2008; Krumpe *et al.*, 2012). These observations have led to the hypothesis that proteins carrying TMDs with the appropriate physico-chemical features can insert spontaneously into the MOM. The MOM would thus represent the default destination for TA proteins with moderately hydrophobic TMD, meaning that these substrates would insert into the MOM if they are not captured by targeting factors directing them elsewhere. This hypothesis is supported also by the observation that some MOM TA proteins (e.g., Fis1) can spontaneously insert into protein-free liposomes (Kemper *et al.*, 2008).

2.2.2.2 Peroxisomes

It has been demonstrated that a sequence of positively charged residues in the extreme C-terminal region are required for targeting of TA proteins to mammalian peroxisomes. Direct insertion of TA substrates into the peroxisomal membrane is mediated by the chaperone PEX19, which in turn binds to its receptor, PEX3, on the peroxisomal surface. As mentioned in section 2.1.3, the PEX19/PEX3 couple is part of the general machinery for the post-translational insertion of peroxisomal membrane proteins and is not dedicated exclusively to TA protein targeting.

Halbach *et al.* (2006) identified two PEX19 recognition sequences in the TMD and lumenal sequence of the mammalian TA protein PEX26, and demonstrated that the interaction between these sequences and PEX19 is required for delivery of PEX26 to peroxisomes. In the absence of the lumenal recognition sequence, PEX26 was inappropriately delivered to mitochondria. Similar Pex19 recognition sequences were identified also in yeast PEX15, a TA protein thought to be the functional homologue of mammalian PEX26. Subsequent studies demonstrated that the presence of positively charged aminoacids was an absolute requirement for the function of these recognition

sequences (Yagita *et al.*, 2013). Another yeast peroxisomal PEX19-dependent TA protein is Fis1, which is involved in peroxisomal division. Interestingly, Fis1 is also targeted to mitochondrial membranes, where it fulfils a similar function. The recognition sequence for PEX9 is at the extreme C-terminus and is not required for mitochondrial targeting (Delille and Schrader, 2008; Mayerhofer P., 2016).

In addition to direct insertion into peroxisomal membranes, ER-to-peroxisome trafficking is known to occur (Tabak *et al.*, 2013). It was found that the correct localisation of overexpressed PEX15 to peroxisomes is GET-dependent. The GET system contributes to the biogenesis of PEX15, by guiding its insertion into the yeast ER, from where it is transported to peroxisomes. Interestingly, yeast cells lacking the GET-machinery still do contain functional peroxisomes, thereby suggesting that PEX15 can be additionally targeted to peroxisomes by a GET-unrelated mechanism, as described in the preceding paragraph (Mayerhofer P., 2016).

In summary, mammalian and yeast TA PMPs may follow two distinct routes for post-translational insertion into peroxisomes: In yeast, TA PMP are predominantly, however not exclusively, targeted through the ER to peroxisomes via the GET-machinery, whereas mammalian TA PMPs probably prefer the direct pathway for insertion into the peroxisomal membrane in a PEX19/PEX3-dependent process. Similar to yeast, the plant TA peroxin APX is also targeted through the ER to peroxisomes. Besides the fact that the sorting signal for APX resides within its C-terminal tail, the implication of the GET pathway in APX trafficking has not been confirmed (Mayerhofer P., 2016).

2.2.2.3 Plastids

Chloroplast TA proteins do not seem to share any conserved sequence for targeting specificity. A noticeable feature of these proteins is that the hydrophobicity value of TMDs of chloroplast TA proteins appears to vary significantly compared to that of mitochondrial TA proteins, which contain a moderately hydrophobic TMD (see section 2.2.2.1). As in mitochondrial TA proteins, basic residues are present on both sides of the TMD to produce a positive net charge (Lee *et al.*, 2014).

TA protein targeting to plastids has been studied for a Chloroplast Outer Envelope (COE) isoform of cyt b5 (AtCb5-6), for the GTPase import receptors Toc33 and 34 and for a COE protein (OEP9) of unknown function. The latter three TA proteins were shown to depend on a chaperone, AKRA2A, previously implicated in post-translational targeting of other types of outer envelope proteins (Dahnoa *et al.*, 2010). However, the targeting signal of Toc33/Toc34 is different from that of OEP9. In the case of OEP9, the targeting signal consists of a 32 amino acid-long hydrophilic chloroplast targeting sequence (CTS) and the TMD. Toc33 and Toc34 also have single TMDs at their C-terminal ends, followed by CTS. However, their targeting to the COE depends on almost the entire protein sequence rather than TMD and CTS, raising the possibility that the targeting of TA proteins to plastids may not solely depend on AKRA2A (Lee *et al.*, 2014).

2.3   Spontaneously inserted tail-anchored proteins

As described in section 2.2.2.1, studies on MOM targeting of TA proteins revealed that a number of MOM substrates are capable of inserting spontaneously into protein-free liposomes in the absence of any membrane or cytosolic protein (Kemper *et al.*, 2008). This capacity is thought to be related to the moderate hydrophobicity of MOM TA proteins, a feature that would allow them to remain soluble in the aqueous environment without the help of chaperones (reviewed in Borgese *et al.*, 2007; Borgese and Fasana, 2011). Most ER-targeted TA proteins require instead chaperone-mediated delivery (section 2.2.1), however, cell-free studies have revealed that a few of them share with MOM TA proteins the capacity for spontaneous insertion into liposomes (Brambillasca *et al.*, 2005; Colombo *et al.*, 2009). Like MOM TA proteins, these are characterised by TMDs of moderate hydrophobicity. Two investigated spontaneously inserting ER TA proteins are described in the last section of this introduction.

2.3.1 Cytochrome b5

Cytochrome b5 (b5) is a small heme-protein, composed of a cytoplasmic domain of 90 amino acids and a C-terminal membrane anchor. Mammalian tissues express an ER-bound cytochrome b5 and a mitochondrial outer membrane isoform, which are the products of 2 different genes. The 2 cyt b5 isoforms are located exclusively, or nearly exclusively, on a single target membrane (D'Arrigo *et al.*, 1993; De Silvestris *et al.*, 1995). Both b5 isoforms are electron carriers, which transfer electrons from a dedicated reductase (NADH-cytochrome b5 reductase) to a number of acceptors. In the ER, b5 is mainly involved in lipid metabolism (Daily and Strittmatter, 1980; Fukushima *et al.*, 1981), whereas the mitochondrial form is capable of regenerating reduced ascorbate from the oxidised, free radical form (Ito *et al.*, 1981).

Mammalian cytochrome b5 is the most thoroughly investigated TA protein capable of unassisted insertion. In studies from our laboratory, it was demonstrated that b5, translated *in vitro* in rabbit reticulocyte lysate (RRL) can translocate its C-terminus across the lipid bilayer *in vitro* in the absence of any membrane or cytosolic protein, and that these do not even facilitate the insertion process (Brambillasca *et al.*, 2005 and 2006; Colombo *et al.*, 2009). Interestingly, however, the presence of even low concentrations of cholesterol in the vesicles inhibits the unassisted integration process (Brambillasca *et al.*, 2005).

Synaptobrevin 2 (Syb2) is a well-known TA substrate incapable of unassisted insertion. In the study of Brambillasca *et al.*, 2006, the substitution of b5's TMD with the one of Syb2 converted b5 into an assisted substrate. Furthermore, point mutations decreasing or increasing TMD hydrophobicity of Syb2 and b5 respectively favoured or inhibited their unassisted insertion. Thus, the feature of b5 responsible for its capacity to insert without assistance is the moderate hydrophobicity of the TMD.

It was found many years ago that the tail region is responsible for discrimination between ER and MOM and, that the extreme C-terminal polar sequence, plays a crucial role in this process, with basic residues favouring targeting to the MOM (De Silvestris *et al.*, 1995). Indeed, mutation of the C-terminal polar domain of the ER targeted protein (to which we refer as b5-ER) to contain a net positive charge results in a protein (b5-RR) that is

specifically targeted to mitochondria *in vivo* (Cartoon 10). Yet, *in vitro*, this mutant inserts into ER microsomes as efficiently as its wild-type counterpart (Borgese *et al.*, 2001). This means that the unassisted insertion *in vitro* may mask chaperone-mediated targeting occurring *in vivo.* Additional possibilities that might be considered are pre-translational targeting phenomena, i.e., localization of mRNA's coding for differently localised TA proteins in close proximity to the target organelle, or post-insertion phenomena, i.e., initial indiscriminate insertion of a TA protein into all permissive membranes, followed by its rapid degradation in the inappropriate organelle. Indeed, a cytosolic factor (Msp1/ATAD1) was demonstrated to route erroneously inserted proteins from the MOM to degradation (Chen *et al.*, 2014; Okreglak and Walter, 2014). At least for b5 localisation, however, we can exclude both mRNA localisation and protein degradation, because: first, microinjected b5 protein is correctly targeted to the ER in cultured cells (Cartoon 11), indicating that the targeting information is in the protein and not in the mRNA; additionaly, since b5 localisation was assessed on cells fixed only after 15 minutes after microinjection, which makes unlikely that complete degradation of mislocalised protein would occur in this short time span. This conclusion is strengthened by the studies in *S. cerevisiae*, where b5 carrying an epitope with a consensus for N-glycosylation, is completely glycosylated after a pulse of only 5 min, indicating that it is all in the ER. It is unlikely that in this short time span, the protein could be inserted into other membranes and then be completely degraded (Yabal *et al.*, 2003).
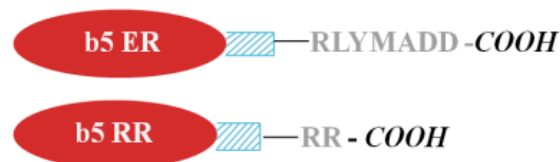
Cartoon 10: Representation of b5-ER and b5-RR. The two proteins are identical in the catalytic domain (red ovals) and in the TMD (blue hatched rectangles), but differ in the short polar region downstream to the TMD (aminoacids indicated with one-letter code).
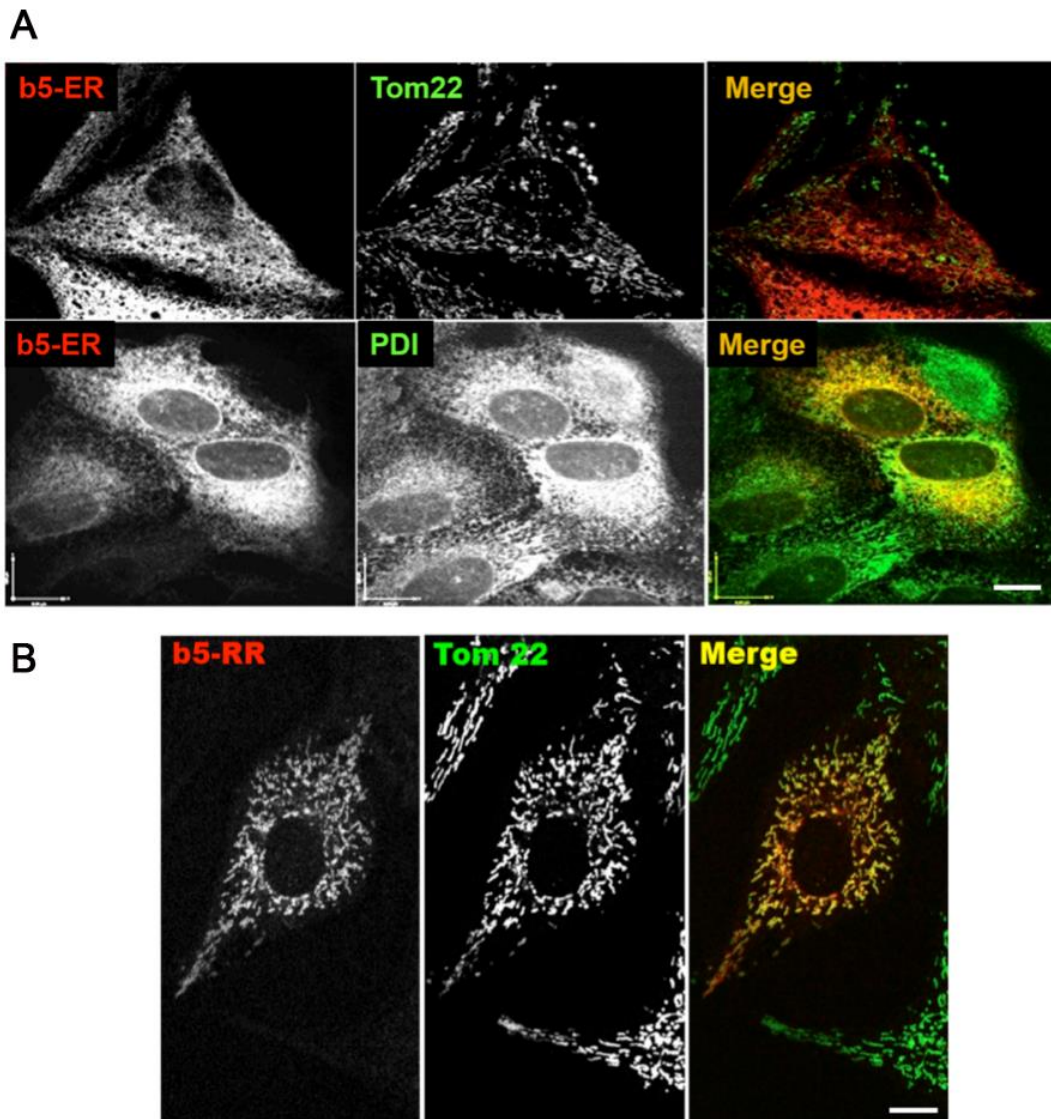
Cartoon 11: Microinjection of recombinant b5-ER and b5-RR in CV1 cells. Purified recombinant b5-ER (A) or b5-RR (B) were microinjected into CV1 cells. 15 minutes after the microinjection the cells were fixed and immunostained with anti-b5 antibodies, anti-Tom22 mAbs (MOM marker), or anti-protein disufide isomerase (PDI – ER marker). Single confocal sections of the single channels and of the merged images are shown; scale bar = 5 μm (Patrizia Cassella, unpublished results).

2.3.2 PTP1B

In a search for other spontaneously inserting TA proteins that target the ER, Brambillasca *et al.* (2006), analised the TMDs of known ER TA proteins and found that protein tyrosine phosphatase (PTP) 1B has a moderately hydrophobic TMD similar to b5. They therefore predicted that this protein might, like b5, be capable of unassisted insertion into liposomes and went on to confirm this expectation experimentally.

PTP1B is an important regulator of diverse cellular signalling networks. For a long time, PTP1B was thought to reside exclusively at the ER; however, it has recently been detected as well in MOM from mammalian cell lines. PTP1B's insertion into the ER has been shown *in vitro* to proceed in the absence of membrane proteins and to be stimulated by Hsp40/Hsc70 chaperones (see section 2.2.1.2; Rabu *et al.*, 2008). The heterologous expression of a fusion protein between mCherry and PTP1B's tail anchor in yeast mutants lacking major conserved ER insertion pathways (GET/TRC, SRP, and Sec62/Sec63 pathways) showed that its ER targeting was poorly affected, providing *in vivo* support for the hypothesis of spontaneous membrane insertion (Fueller *et al.*, 2015).

3. Aim of the thesis

The unique mechanisms by which tail-anchored (TA) proteins achieve their final subcellular localisation have, in the last years, attracted a great deal of interest, and constitute the subject of this thesis. Although the main pathway for targeting to the endoplasmic reticulum (ER) of TA proteins is represented by the TRC40/Get3 pathway, many TA proteins, i.e., those targeted to the mitochondrial outer membrane (MOM) and some ER targeted ones, can insert *in vitro* into pure phospholipid bilayers without assistance from any chaperone. Because *in vivo* they have a precise destination, it is clear that targeting factors must be involved, but these factors have not been identified to date.

The aim of this thesis was to investigate the targeting of TA proteins that are capable of spontaneous insertion into lipid bilayers in cell-free systems. For this purpose, I have used as model cytochrome b5, of which two forms are known: the wild-type protein, directed to the ER (b5-ER), and the b5-RR protein, addressed to the MOM; the two proteins differ only in the last 7 amino acids at the C-terminus.

The project is based on the following previous observations from the work of my laboratory: (i) the two proteins described above are faithfully targeted to the ER or to the MOM in transfected cells, but fail to be specifically targeted in cell-free systems based on the rabbit reticulocyte lysate (RRL); (ii) each of the two recombinant proteins is specifically targeted to the correct organelle when microinjected into the cytoplasm of cultured cells. The latter observation excludes that mRNA localisation or ribosome-associated factors account for the correct localisation *in vivo*, and suggest that cell-free systems, in which only one possible acceptor membrane is provided, do not reflect the complexities of *in vivo* targeting.

Using methods of transfection, RNA silencing, microinjection and import assays with semi-permeabilised cells, I tried to understand the basis of the specificity of the "targeting" of both forms of cytochrome b5, by evaluating the involvement of different pathways and cytosolic chaperones. In summary, the main objective of this project is to define the molecular mechanisms underlying the unique biological properties of this class of proteins: How do TA proteins elect their target membranes? How do these proteins choose between the ER and the MOM?

Due to their essential roles in basic cellular functions and in the progression of diseases, understanding the biogenesis of TA proteins is important. Although my study was carried out on cytochrome b5 as model protein, it is relevant for other physiologically crucial TA proteins, such as those of the Bcl-2 family.

4. Materials and Methods

4.1 Plasmids and recombinant proteins

4.1.1 Constructs

The cytochrome b5 (b5) constructs used in this study were all available in the laboratory, and are schematized in Figure 1A. They were all derived from the plasmid pGb(5)AX, described in Pedrazzini *et al.* (2000). This plasmid contains the coding sequence of rabbit ER b5, subcloned in the *KpnI*, *XbaI* sites of pGEM4, and having a unique *AgeI* site at the border between the regions specifying the TMD and the C-terminal polar residues, and a *HindIII* site immediately downstream to the *XbaI* site. To express this cDNA in mammalian cells, the b5 insert was placed under the cytomegalovirus (CMV) promoter by subcloning it into pCB6 vector (construct 1).

The plasmids pGEX2T, under the inducible promoter Tac, code for GST fusion proteins and so, are suitable for expression and purification of recombinant proteins from bacteria (constructs 2 - 4). The pGEM4 plasmids coding for b5-ER with a C-terminal opsin tag (b5-ops - construct 5), for b5 with the TMD of synaptobrevin 2 and a C-terminal opsin tag (syb2-ops - construct 7), and for PTP1B with a C-terminal opsin tag (PTP1B-ops - construct 6), under the SP6 promoter, were described previously (Brambillasca *et al.*, 2006; Colombo *et al.*, 2009). These constructs contain at the C-terminus a tag derived from the N-terminal sequence of bovine opsin; this tag contains a site for N-glycosylation, which allows the detection of translocation of the protein into the ER lumen.

The pQE80-MBP-WRBcc plasmid (construct 10), which codes for a fusion protein between maltose binding protein (MBP) and the coiled-coil cytosolic domain of WRB, was a kind gift from Fabio Vilardi (University of Goettingen - Favaloro *et al.*, 2010, Vilardi *et al.*, 2011). The pQE80-MBP-WRBcc R77E plasmid (construct 11), a mutant version of WRBcc in which an arginine is substituted by a glutamate, was a kind gift from Stephen High (University of Manchester). Two other constructs used for transfection as ER markers were pRFP-KDEL (construct 8), obtained from Erik Snapp (Janelia Research Campus) and pEGFP-E-Syt1 (extended-Synaptotagmin-1 – construct 9), obtained from Pietro de Camilli (Yale University School of Medicine).

4.1.2 Transformation of bacteria

To prepare bacteria competent to incorporate exogenous DNA, the method developed by Hanahan (1983) was followed. Competent bacteria were stored frozen, and thawed and kept on ice for 10 minutes before use. In the meantime, 2-20 ng of plasmid DNA were diluted in 10 μl of water in transformation tubes. 100 μl of bacteria were added to the DNA solution, and the sample was left on ice for 30 minutes. Next, they were subjected to a heat shock step for 90 seconds at 42°C, thus allowing the DNA to enter the cells. After a quick cooling step on ice, 800 μl of SOC medium [SOB (2% Tryptone, 0.5% Yeast extract, 10mM NaCl, 2.5mM KCl) supplemented with 20 mM glucose and 10 mM $MgCl_2$] were added and the bacteria were incubated for 1h at 37°C with shaking to allow recovery. Finally, they were plated on a petri dish supplemented with the appropriate selection antibiotic and allowed to grow overnight at 37°C.

4.1.3 DNA extraction

Different procedures are available for isolating DNA plasmids grown from bacteria colonies. In this work, I carried out medium-scale isolation of plasmid DNA (MIDI preparations). The MIDI were performed according to QIAGEN Plasmid Midi kit:

- 2 ml of LB (Luria Bertani medium: 10g Tryptone, 10g NaCl, 5g Yeast extract, pH 7.0), containing the appropriate antibiotic were inoculated with a single bacterial colony and incubated overnight at 37°C with vigorous shaking;
- On the following day, 400 ml of LB medium were inoculated with 400 μl of the overnight culture, always in the presence of the antibiotic, and the sample was shaken overnight at 37°C. At this point, there were enough bacteria to allow a proper extraction of large amount of plasmid DNA;
- Bacteria were centrifuged at 4°C, for 30 minutes at 4000 rpm;
- The pelleted cells were resuspended in 4 ml of Buffer P1 (Resuspension Buffer);

- 4 ml of Buffer P2 (Lysis Buffer) were added and the tube was incubated at room temperature for 5 minutes. This step is to lyse cells and denature DNA;

- 4 ml of Buffer P3 (Neutralization Buffer) were added and immediately the lysate was mixed by inverting the tube 4-6 times. This step is to neutralize the effects of the lysis buffer and to generate a K^+-SDS precipitate. Under these conditions, the small plasmid DNA is renatured, while the large chromosomal DNA co-precipitates with the K^+-SDS;

- After 15 minute of incubation on ice, the sample was centrifuged at 12000 rpm at 4°C for 40 minutes to eliminate genomic DNA and cell debris;

- The supernatant was applied to the Qiagen-cartridge, which was previously calibrated with QBT Buffer;

- The cartridge was washed 2 x 10 ml with Buffer QC (Wash Buffer) and the DNA was eluted with 5 ml of Buffer QF (Elution Buffer);

- The DNA was precipitated by adding 3.5 ml room-temperature isopropanol to the eluted DNA and it was immediately centrifuged at 8000 rpm for 40 min at 4°C;

- The DNA pellet was washed with 2 ml of room temperature (RT) 70% ethanol, and centrifuged another 10 minutes;

- The pellet was air-dried, redissolved in TE buffer (10 mM Tris-Cl, pH 7.5 and 1 mM EDTA), analysed and stored at -20°C.

The purity and concentration of the preparations was checked by UV absorption and agarose gel electrophoresis.

### 4.1.4 Protein expression and purification from bacteria

- **Purification of b5-ER, b5-RR and b5-ops**

To express and purify b5-ER and b5-RR proteins in bacteria, the Glutathione S-transferase (GST) Gene Fusion System was used, following the manufacturer's instructions (GE Healthcare). GST-tagged proteins are constructed by inserting a gene or gene fragment into pGEX vectors. Expression is under the control of the tac promoter, which is induced by

the allolactose analogue isopropyl β-D-thiogalactoside (IPTG). All pGEX vectors are also engineered with an internal lacIq gene. The lacIq gene product is a repressor protein that binds to the operator region of the tac promoter, blocking the expression of the downstream genes. When IPTG is added, it binds to lacIq and cancels the repression on the promoter, thus triggering the transcription of the downstream gene (Cartoon 12).
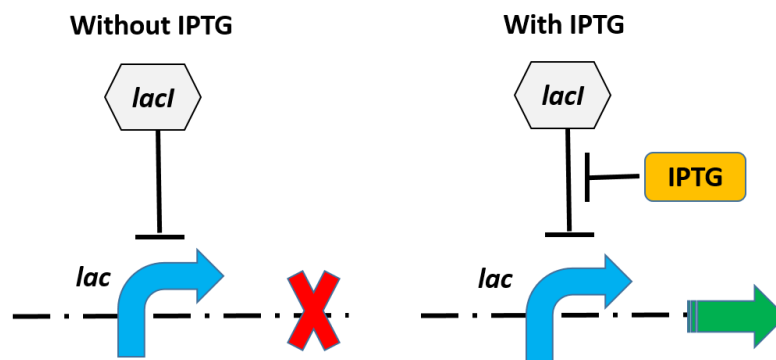
Cartoon 12: Mechanism of induction by IPTG.

The purification of GST-tagged proteins is based on the affinity of GST to the glutathione ligand coupled to a matrix, followed by proteolytic cutting with thrombin, which recognises a cleavage site present between the sequence of the GST and that of b5 (Cartoon 13).

BL21 bacteria, transformed with plasmids coding for GST fusion proteins with the different b5 forms, and induced for 4 hours with 0.5 mM IPTG, were collected from 200 ml cultures and lysed with 16 ml of buffer containing 2% Triton-X100, 150 mM NaCl, 25 mM Tris-Cl pH 7.4, 0.2 mg/ml lysozyme, 10 mg/ml DNase, plus a protease inhibitor mixture. The sample was incubated at room temperature until its viscosity started to disappear; at this point, 10 mM EDTA was added and the incubation was continued for another hour at room temperature. Then, the lysate was clarified by centrifugation (10 000 × g for 40 minutes at 4°C) and the soluble fraction was incubated for 1 hour at 4°C on a rotating wheel with GSH-Sepharose 4B beads (GE-Healthcare). After removing unbound material, the beads were incubated with a solution containing 2 mM ATP, 10 mM $MgSO_4$, 50 mM Tris-Cl pH 7.4 for 10 minutes at 37°C, to remove associated chaperones. The beads were then washed 6 times

with cold PBS. The b5 constructs were then released by incubation for 2 hours at room temperature with 4 units (U) of thrombin (Sigma). The yield of the thrombin cleaved protein was evaluated by the BCA (bicinchoninic acid) assay (Euroclone) or by comparison of its Coomassie Brilliant Blue (CBB, Bio-Rad) staining with that of known amounts of bovine serum albumin (BSA, Thermo Fisher) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Cartoon 13: GST purification system. The GST fusion protein binds to the Glutathione-Sepharose 4B resin (GS4B) and, after removing the unbound fraction, thrombin is used to cut GST from the protein.

- **Purification of MBP-WRBcc and MBP-WRBcc R77E**

The plasmid MBP-WRBcc was obtained from Fabio Vilardi and has been described previously (Favaloro *et al.*, 2010; Vilardi *et al.*, 2011). WRBcc (WRB coiled-coil) corresponds to the cytosolic domain of WRB. The plasmid MBP-WRBcc encodes WRBcc merged to MBP (Maltose binding protein), cloned into the vector pQE80 (Qiagen). The plasmid MBP-WRBcc R77E (Stephen High, University of Manchester), codes for a mutant, non-functional version of WRBcc in which an arginine is substituted by a glutamate. Like in the case of the pGEX plasmids, the MBP fusion proteins are under the control of an IPTG-inducible promoter. In this case, however, the affinity purification involves the binding of MBP to an amylose-containing resin. The protein of interest can then be cleaved from MBP thanks to the presence of a TEV cleavage site.

MBP-WRBcc and mutant MBP-WRBcc were purified from the soluble fraction of a sonicate obtained from BL21 cells induced by IPTG. The BL21 cells, obtained from 50 ml of

culture, were suspended in 2 ml of sonication buffer (200 mM NaCl, 20 mM Tris-Cl pH 7.5, 1 mM EDTA, 1 mM DTT, 1 mM PMSF), and the cells were lysed by sonication. The soluble fraction was diluted 1:5 in the same buffer, and incubated for 2 hours at 4°C with 3 ml of packed Amylose resin (New England Biolabs). After this, the protein was eluted with a 10 mM maltose solution (Sigma), and concentrated with the Amicon 3K Filter System (Merck). Also in this case the purification was checked by CBB staining of SDS-polyacrylamide gels, and quantified by the BCA assay. To cleave the WRBcc from MBP, the eluted fraction was incubated overnight at 4°C with GST-tagged TEV protease (Sigma), based on the concentration of each substrate (1 mg of TEV/100 mg of protein). The protease was then eliminated by incubation of the digested sample with Glutathione-Sepharose 4B.

4.2 Cell culture, transfection and microinjection

HeLa cells, a human epithelial cell line derived from cervical cancer, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin antibiotic mix and 1% L-glutamine (all from Thermo Fisher) at 37°C and 5% CO_2.

4.2.1 Transient DNA transfection

In transient transfection, the introduced nucleic acid exists in the cell only for a limited period of time and is not integrated into the genome. I used the jetPEI system (Polyplus Transfection) for transient transfection. jetPEI compacts DNA into positively charged particles capable of interacting with anionic proteoglycans on the cell surface. Upon binding to the cell membrane, the complexes are internalised via endocytosis. Once inside the endosomal compartment, the DNA is protected from degradation by jetPEI. This is in part due to the unique property of this polymer to act as a "proton sponge", which in turn buffers the pH within the endosome. This mechanism ultimately leads to rupture of the endosome and release of the DNA and the complexes into the cytoplasm, thereby allowing nuclear transport for subsequent transcription.

For transfection with jetPei, I followed the manufacturer's instructions. Briefly, cells were plated 24h before transfection in 6-multiwell plates to allow growth to 50% confluence. Prior to transfection, the medium was changed to remove cell debris. For each well, two solutions were prepared: the first one containing 2 µg of DNA, the second one 6 µl of jetPEI; both were diluted to 100 µl of 150 mM NaCl. After mixing, the jetPEI solution was added to the one containing the DNA. This mixture was stored at room temperature for 20 minutes and then added to cells. The cells were analysed 24 h later.

4.2.2 RNA silencing

RNA interference (RNAi) is an effective way to knock down gene expression to study protein function in a wide range of cell types. For my experiments, I used short double stranded RNA oligonucleotides, 21 nucleotides in length; this length corresponds to the size of endogenous interfering RNAs generated from pre-microRNA transcripts by an enzyme called Dicer. The RNA duplex is designed to target the chosen mRNA. The antisense strand of the siRNA duplex becomes part of a multi-protein complex, or RNA-induced silencing complex (RISC), which then identifies the corresponding mRNA and cleaves it at a specific site, by endonucleases called argonaute proteins. Next, this cleaved message is targeted for degradation, which ultimately results in the loss of protein expression.

For silencing procedures, cells plated in DMEM without antibiotics at approximately 20% confluence were transfected with RNAi duplexes with the use of RNAiMAX Lipofectamine reagent (Thermo Fisher). For transfection, two solutions were prepared: one containing the appropriate volume of siRNA (starting from stock solutions of 10 µM), and the other one containing Lipofectamine in a ratio 1:1 (1 µl of lipofectamine per 1 µl of siRNA); both diluted in Optimem (Thermo Fisher). After mixing, the siRNA solution was added to the one containing the Lipofectamine. This mixture was stored at RT for 20 minutes and finally added to cells. Parallel cultures were transfected with equal concentrations of Silencer Select negative control RNA (Thermo Fisher). Six hours after transfection, the medium was replaced with complete growth medium; cells were collected and analysed after 48h (for WRB and SGTA silencing) or 72h (SND2 silencing) after transfection. Specifically for Sec61, a

second transfection was done 24 h after the first one and the cells were collected 48 h after the second transfection.

- WRB was silenced with two different siRNAs (Ambion Silencer Select siRNAs), each at a concentration of 5nM. The sequence of the sense strand is given: s14905 (WRB siRNA#1: 5' CAGUCAACAUGAUGGACGAtt 3'); s14906 (WRB siRNA#2: 5' GGGUGAUAAGUGUCGCUUUtt 3');

- SGTA was silenced with two different siRNAs (Ambion Silencer Select siRNAs), each at a concentration of 10 nM. The sequence of the sense strand is given: s12783 (SGTA siRNA#1: 5' GCUUCGAACCUAAUGAACAtt 3'); s12782 (SGTA siRNA#2: 5' GCCGUGCAUUUCUACGGAAtt 3');

- Sec61 alpha subunit was silenced with two different siRNAs (Ambion Silencer Select siRNAs) each at a concentration of 10 nM. The sequence of the sense strand is given: s30355 (Sec61 siRNA#1: 5' CGCACGUUCUUACCCAGUUtt 3'); s26722 (Sec61 siRNA#2: 5' GAGUGAUUCUAGCCUCUAAtt 3');

- SND2 protein was silenced with four different siRNAs (Qiagen), each at a concentration of 20 nM. The sequence of the sense strand is given: SND2 siRNA#1, 5'CCUUAAGGAUGUGAUCCUAtt3'; SND2 siRNA#2, 5' CUAUAGGGUCGUU-GAAUAAtt3'; SND2 siRNA#3 5' GGGCAAAGUGGGCACGAGAtt3'; SND2 siRNA#4 5'GGGUAUACUUAUACUCUAUtt 3'.

4.2.3 Microinjection

Microinjection was performed with an Eppendorf 5200 microinjector by applying a pressure of 80-90 hPa, which injected plasmids (in water solution at a concentration of 100-200 ng/µl) into the nucleus of HeLa cells cultured at 80% of confluence. The microinjected cells were incubated 15 minutes at 37°C and then fixed with 4% paraformaldehyde for 20 minutes.

4.3 Antibodies

In this work, antibodies were used in both biochemical and immunofluorescence assays. The protocols used require an incubation with a primary antibody followed by an incubation with a secondary antibody. For immunofluorescence (IF) experiments, the secondary antibodies are conjugated with a fluorophore, while for Western blot (WB) experiments, the secondary antibodies are conjugated with peroxidase or with infrared fluorophores.

4.3.1 Primary antibodies

Primary antibodies are immunoglobulins raised against an antigenic target of interest (a protein, peptide, carbohydrate, or other molecule) and are typically unconjugated (unlabelled). Primary antibodies that recognise and bind with high affinity and specificity to unique epitopes across a broad spectrum of molecules are available as high specificity monoclonal antibodies and/or polyclonal antibodies. These antibodies are useful also to detect modifications mediated by processes such as phosphorylation, methylation or glycosylation. The primary antibodies used in this study are summarised below:

Antigen	Type of antibody	Origin	Application
Opsin R2-15	Mouse mAb	Paul Hargrave	WB/IF
Cytochrome b5	Rabbit polyclonal	Produced in the lab	WB/IF
WRBcc	Rabbit polyclonal	Synaptic Systems	WB
TRC40	Rabbit polyclonal	Produced in the lab	WB
PDI	Mouse mAb	Enzo Life Sciences	WB
MFF	Mouse mAb	Proteintech	IF
Calnexin	Rabbit polyclonal	Ari Helenius	WB
Tom-20	Mouse mAb	Abnova	IF
Sec61α	Rabbit polyclonal	Richard Zimmermann	WB
SND2	Rabbit polyclonal	Richard Zimmermann	WB
SGTA	Chicken polyclonal	Stephen High	WB/IF

4.3.2 Secondary antibodies

A secondary antibody binds to primary antibodies or antibody fragments. They are typically labelled with probes that make them useful for detection, such as peroxidase or fluorophore. The secondary antibodies used in this study are summarised below:

Species reactivity	Conjugation	Origin	Application
Rabbit	Peroxidase	Sigma	WB
Rabbit	IRDye 800CW	LI-COR	WB
Rabbit	Alexa Fluor 488	Life Technologies	IF
Rabbit	DyLight 549	Jackson	IF
Chicken	IRDye 800CW	LI-COR	WB
Mouse	Peroxidase	Sigma	WB
Mouse	IRDye 680RD	LI-COR	WB
Mouse	Alexa Fluor 647	Life Technologies	IF
Mouse	Alexa Fluor 633	Life Technologies	IF
Mouse	DyLight 549	Jackson	IF

4.4 Import assays of tail-anchored proteins

To investigate TA protein targeting, I used a system based on semi-intact (also called semi-permeabilised) cultured cells (Setoguchi *et al.*, 2006). In this system (Figure 1B), adherent cells are permeabilised by low concentrations of digitonin, which, because of its affinity for cholesterol, disrupts the permeability barrier of the plasma membrane, while leaving intracellular membranes intact. In this way, the interior of cells has access to added macromolecules. The semi-intact cells are washed, to remove the cytosol, which can then be replaced by an exogenous source of cytosol, manipulated as desired. In my experiments, I used the rabbit reticulocyte lysate (RRL) as source of cytosol. TA protein substrates were introduced into the system either as recombinant proteins or as the products of *in vitro* translation.

4.4.1 Preparation of RRL

RRL, purchased from Green Hectares, was treated with Hemin solution (to have a solution of 0.65 mg/ml, I first dissolved 6.5 mg of hemin in 250 µl 1N KOH; the volume was then brought to 10 ml to obtain a solution containing 10 mM Tris pH 8.0, 89 % (v/v) ethylene glycol, and 25 mM HCl; the solution was frozen in liquid nitrogen and stored at -80 °C). Hemin is added because it suppresses hemin-regulated inhibitor kinase, which, by phosphorylating initiation factor elF2α, inhibits the initiation of translation; in the absence of hemin, protein synthesis in the RRL will cease after a short period of incubation. It should be noted that the RRL was not treated with nuclease to remove endogenous mRNA. In this way, the presence of EGTA to inactivate the nuclease is avoided, and thus the endogenous levels of Ca^{2+} are kept. When thawed, the following components were added (final concentrations specified): 0.07 mM of each amino acid, 22.75 mM Hepes, 18.63 mM KOH, 17.5 mM ATP, 1.75 mM GTP, 17,5 mM creatine phosphate, 87.6mM KOAc, 2.2 mM $MgCl_2$, 70 ng/µl creatine kinase, 2 mM glutathione and 1 µM taxol.

4.4.2 *In vitro* transcription and translation

b5-ops, syb2-ops and PTP1B-ops in pGEM4 were transcribed by SP6 polymerase (Promega) for 1 hour at 37°C (5 µg of DNA per 20 µl of transcription), and the resulting synthetic RNA was translated for 1 hour at 32°C in RRL (20 µl of the transcription reaction plus 100 µl of RRL mixture), treated as described in the preceding section. In both processes, RNasin (Promega) was added to avoid RNA degradation. In some experiments (the ones with the PTP1B protein), the translation was done in the presence of 1 mCi/ml ^{35}S-methionine (Perkin Elmer) omitting the addition of non-radioactive methionine to the mixture. For all the others, the translation was carried out with unlabelled methionine, and the translation products were revealed by western blotting with anti-opsin antibodies. In all cases, translation was blocked by addition of 300 µg/ml cycloheximide (Sigma), and ribosomes were removed (55000 rpm for 1 hour at 4°C) before carrying out post-translational insertion assays.

4.4.3 Permeabilisation of HeLa cells and incubation with TA substrates

The protocol was adapted from Setoguchi *et al.* (2006). This procedure allows the evaluation of the localisation of proteins in a system intermediate between *in vivo* cultured cells and a cell-free system.

HeLa cells were seeded onto 22x22 mm or Ø 15 mm coverslips in 6 or 12-multiwell plates and grown to ≥80% confluence. Cells were washed twice with import buffer (0.25 M sucrose, 2.5 mM $Mg^{2+}(OAc)_2$, 25 mM KCl, 1 µM taxol, 20 mM Hepes-K^+ pH 7.4), incubated at 26°C for five minutes in 800 µl the same buffer containing of 25 µM digitonin (Calbiochem), washed two times with import buffer and then exposed to 300 µl (or 120 µl for Ø 15 mm coverslips) of KHM buffer (110 mM K^+OAc, 20 mM Hepes-K^+ pH 7.4, 2 mM $Mg^{2+}(OAc)_2$, 1 µM taxol) or RRL containing either the *in vitro* translated (b5-ops, syb2-ops, PTP1B-ops) or the recombinant protein (0.5 µg of b5-ER, b5-RR or b5-ops). After incubation at 26°C for 1 hour, the cells were washed 3 times with KHM buffer. Targeting of TA proteins to the ER or mitochondria was assessed by IF analysis of the cells fixed with 4% paraformaldehyde (section 3.6). Alternatively, integration of TA proteins into the ER of semi-intact cells was monitored by evaluating the percentage of glycosylation of the C-terminal opsin epitope. To this end, cells were lysed with 100 µl of lysis buffer (1% sodium dodecyl sulfate (SDS), 0.1 M Tris-Cl pH 8.9 supplemented with protease inhibitors) and heated for 2 min at 90°C.

4.4.4 Pharmacological treatment of the RRL

After translation of the investigated TA substrate, the RRL was treated with one of the inhibitors listed in the table below. The Hsp70 inhibitors, obtained from Jeffrey Brodsky (University of Pittsburgh), are described in Fewell *et al.* (2004) and Wright *et al.* (2008). WRBcc corresponds to the coiled-coil domain of WRB and acts as decoy receptor for TRC40 (Vilardi *et al.*, 2011, see section 3.1.4). The table also lists the conditions of the treatment and the solvent in which each substance was dissolved. Controls were always carried out with equal volumes of the vector.

At the end of the treatment, the RRL containing the *in vitro* translated TA protein was added to adherent semi-intact cells. When the recombinant protein was used, it was added at the end of the treatment and before incubation with cells.

Compound	Source	Concentration and solvent	Time of treatment	Action
MAL2-11B	Jeffrey Brodsky	100 µM in DMSO	15 min RT	Hsp70 inhibitor
MAL3-51	Jeffrey Brodsky	100 µM in DMSO	15 min RT	Hsp70 false inhibitor
DMT 002220	Jeffrey Brodsky	100 µM in DMSO	15 min RT	Hsp70 inhibitor
DMT 003024	Jeffrey Brodsky	100 µM in DMSO	15 min RT	Hsp70 inhibitor
Geldanamycin	Calbiochem	10 µM in DMSO	15 min RT	Hsp90 inhibitor
WRBcc	Fabio Vilardi	5 µM in KHM	15 min RT	Inhibitor of TRC40 pathway
WRBcc R77E	Stephen High	5 µM in KHM	15 min RT	TRC40 false inhibitor
Eeyarestatin I (ES I)	Sigma	10, 20 and 100µM in DMSO	1h on ice	Inhibitor of ERAD
Eeyarestatin 35	Stephen High	10, 20 and 100 µM in DMSO	1h on ice	Inactive analogue of ES I
DBeQ	Sigma	20 µM in DMSO	1h on ice	p97 inhibitor

4.4.5 TRC40 depletion

Protein-G-Sepharose (150 µl) was incubated with 150 µl anti-TRC40 antibody (20 mg/ml total Immunoglobulin fraction generated by NH_4sulfate precipitation,) for 1 hour at 4°C. After removing the supernatant, the resin was incubated with 350 µl of RRL for 1 hour at

4°C, and the depleted lysate was recovered after removal of the beads by centrifugation. As control, a solution of unrelated IgG was used instead of the antibody (mock depletion). Depletion of TRC40 was checked by western blotting.

4.4.6 ATP depletion

ATP was depleted from RRL with a hexokinase/glucose trap (adapted from Yabal *et al.*, 2003). The ATP-dependent phosphorylation of glucose to form glucose 6-phosphate is the first reaction of glycolysis, and is catalysed by the enzyme hexokinase, resulting in the depletion of all available ATP. The RRL was first prepared with all components except creatine phosphate, ATP, GTP and creatine kinase. Then, a solution containing 30 mM glucose and 2.5 µg/ml hexokinase (both from Sigma) was added.

4.5 Biochemical analyses

4.5.1 Carbonate extraction

A simple method for determining if an integral membrane protein has achieved stable insertion into the bilayer is alkaline extraction. Commonly, preparations are subjected to a sodium carbonate solution (pH 11-12). Upon treatment with sodium carbonate, protein-protein interactions are disrupted, concomitantly stripping attached peripheral proteins, while protein-lipid interactions remain and the bilayer is otherwise intact (Fujiki *et al.*, 1982). For carbonate extraction analysis, cells were collected with cold PBS, and homogenised by repeatedly passing the cell suspension through a 26G × ½'' needle; the total homogenate was then incubated with and equal volume of 0.2 M Na_2CO_3 for 30 minutes on ice. An aliquot was set aside as input, and the rest of the sample was ultracentrifuged at 60.000 rpm (rotor TLA 100.3, Beckman) for 1 hour at 4°C, in order to separate carbonate-soluble and insoluble proteins. The pellets were resuspended in the original volume of 0.1 M Na_2CO_3, and equal aliquots of pellet and supernatant were analysed by SDS-PAGE/WB.

4.5.2 Treatment with glycanase

The enzyme N-glycanase F (PNGase F, New England Biolabs) was used to remove N-linked glycans from our constructs, according to the instructions of the manufacturer. *In vitro* synthesized or recombinant proteins were first immunoprecipitated. To this end, 60 µl of sample were diluted with eleven volumes of IP buffer (100 mM NaCl, 50 mM Hepes-K pH 7.4 and 1% Triton X-100); the sample was then supplemented with 40 µl Protein-G-Sepharose (50 % slurry) and 8 µg IgG/ml of anti-opsin mAb. After incubation overnight at 4°C in a rotating wheel, the beads were collected by centrifugation and washed once with IP buffer and once with H_2O. 20 µl of 1× Denaturing Buffer (0.5% SDS, 40 mM DTT) was added and the glycoprotein was denatured by heating the sample at 95°C for 5 minutes. After centrifugation, the eluate was divided in 2 aliquots, one for digestion and one for control. To 10 µl of eluate, 2 µl 10× G7 Reaction Buffer (500 mM sodium phosphate pH 7.5), 2 µl 10% NP40 and 6 µl H_2O were added. PNGase F (1 µl) was added only to the digestion aliquot and both samples were incubated at 37°C for 1 hour. The reduction in molecular weight caused by deglycosylation was analysed by SDS-PAGE/WB (section 3.5.5).

4.5.3 Determination of glycosylation with Concanavalin beads

This technique was used to analyse the glycosylation of PTP1B, since the glycosylated protein migrates very close to the non-glycosylated one. Concanavalin A (Con A) is a tetrameric metalloprotein isolated from *Canavalia ensiformis* (jack bean). Con A binds molecules containing α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related residues. Concanavalin A coupled to Sepharose is routinely used for separation and purification of glycoproteins, polysaccharides and glycolipids.

To perform this protocol, cells were recovered with Binding Buffer (20 mM Tris-HCl pH 7.4, 0.5 M NaCl, 1% Triton and protease inhibitors) and, after elimination of nuclei, the supernatant was incubated with ConA Sepharose resin (GE-Healthcare) overnight in a rotating wheel at 4°C, in a ratio of 15 µl packed ConA beads per 150 µg of protein. The mixture was then centrifuged; the resulting supernatant, corresponding to the

unglycosylated fraction, and the beads, containing the glycosylated proteins, were analysed by SDS-PAGE.

4.5.4 SDS-PAGE and Western Blot

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique widely used in biochemistry to separate proteins according to their molecular mass. This is because the ionic detergent SDS denatures and binds to proteins to make them evenly negatively charged. Thus, when a current is applied, all SDS-bound proteins in a sample will migrate through the gel from the negatively charged electrode (cathode) towards the positively charged one (anode). Besides the addition of SDS, proteins may optionally be briefly heated to near boiling in the presence of a reducing agent, such as mercaptoethanol, which further denaturates the proteins by reducing disulfide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure.

In this study, before loading, samples were boiled for 2 minutes after adding 1/2 of volume of Denaturation Buffer (DB) 3X (3% β mercaptoethanol, 25.5% glycerol, 6% SDS, 60 mM Tris-HCl pH 6.8, 0.03% bromophenol blue). Polypeptides were then separated on polyacrylamide gels of appropriate concentration, depending on the analysed protein. Separated polypeptides were visualised by CBB staining. When the *in vitro* translation with ^{35}S-methionine was analysed, the resulting radioactive gels were stained with CBB, dried and imaged with the Storm phosphoimager (GE-Healthcare). Band intensities were quantified with Image Quant TL 7.0 software.

More commonly, recombinant or *in vitro* synthesized polypeptides were visualised by Western Blotting (WB). This technique uses specific antibodies to identify proteins that have been separated by gel electrophoresis. With the application of an electrical current, the proteins are transferred from the gel to an adsorbent membrane where they become immobilised as a replica of the gel's band pattern. The membrane can then be further processed with antibodies specific for the target of interest, and visualised using secondary antibodies and detection reagents. In this work, proteins were transferred onto 0.2 μm nitrocellulose membrane (Perkin Elmer). The blots were probed with the indicated primary

antibodies, followed by peroxidase- or infrared- conjugated secondary antibodies (see sections 3.3.1 and 3.3.2). In the first case, bands were visualised with ECL Western blotting detection system (Perkin-Elmer). In the second case, blots were scanned with the Odyssey CLx Infrared Imaging System (LI-COR, Bioscience) and the bands were quantified with Image Studio software (LI-COR, Bioscience).

4.6 Immunofluorescence

The cells grown on glass coverslips were fixed for 20 minutes at RT with 4% paraformaldehyde in 120 mM phosphate buffer pH 7.6. Fixed cells were generally stained with antibodies under high salt conditions (procedures 1 and 2, below). Incubations with antibodies was done by placing the coverslips on top of a small drop of solution, deposited on a layer of parafilm in a humid chamber.

Procedure 1: This was used for the detection of cytosolically exposed epitopes in cells previously permeabilised with digitonin (section 3.4.3). In this case, additional permeabilisation was not required. Formaldehyde was quenched and nonspecific binding sites saturated by incubating the coverslips over a drop of GDB (gelatin dilution buffer: 0.1% gelatin, 40 mM phosphate buffer pH 7.6, 0.45 M NaCl) for 1 hour. The cells were then incubated for 2 hours with primary antibody diluted in GDB, followed by 3 washes in High Salt buffer (HS = 20 mM Tris-HCl pH 7.4, 500 mM NaCl), and incubation with the secondary antibody diluted in GDB for 1 hour. At the end the coverslips are washed 3 times in HS, once with PBS, once with H_2O, mounted with Mowiol (Sigma), and sealed with nail polish.

Procedure 2: This was used for cells not previously permeabilised or to detect epitopes in the lumen of organelles, whose delimiting membrane would not be permeabilised by the previous digitonin treatment. The procedure is identical to procedure 1, except that the GDB is supplemented with 0.3% Triton X100.

Confocal images were acquired using a LSM 510 META (Carl Zeiss) connected to an Axiovert 200M microscope (Carl Zeiss), or with the Confocal Spinning Disk LCI Ultraview (Perkin Elmer) system connected to an Axiovert 200M microscope (Carl Zeiss). In all cases,

the images were acquired using a 63X magnification Plan-Apochromat lens. Single confocal sections are shown in the illustrations, which were prepared with Photoshop software.

4.6.1 Image analysis

Colocalisation of cytochrome b5 with mitochondria or endoplasmic reticulum markers were quantified by Mander's colocalisation coefficient (ImageJ "JACoP" plug-in analysis) (Manders *et al.*, 1992; Bolte and Cordelieres, 2006), which corresponds to the fraction of overlapping pixels between two channels. This coefficient will vary from 0 to 1, the former corresponding to non-overlapping images and the latter reflecting 100% co-localisation between both images.

Before running the JACoP plug-in, the background of each image was subtracted and a region of interest (ROI) for each cell to be analysed was manually drawn.

4.7 Statistical analysis

All quantitative data are presented as mean ± SEM. The number of experiments is indicated in the legend of each figure. When the errors bars are not indicated, it is because the graph represents a single experiment. In the case of image analysis, images were pooled from at least 3 independent experiments. Comparisons between two groups were carried out by paired or unpaired Student's t-test (Excel), as indicated in the figure legends. Multiple comparisons among groups were carried out by One-Way ANOVA (Prism) and Bonferroni's post-test. In the figures, the following symbols are used: ns (not significant), $p > 0.05$; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$.

5. Results

1. Cytosolic factors are required for faithful targeting of b5-ER but not of b5-RR in semi-intact cells.

As described previously in the introduction, each of the two recombinant cytochrome b5 forms, b5-RR and b5-ER, is specifically targeted to the correct organelle when microinjected into the cytoplasm of cells. In addition, the two proteins are faithfully targeted to the ER or to the MOM in transfected cells, but fail to be specifically targeted in cell-free systems based on the rabbit reticulocyte lysate (RRL) (Borgese *et al.*, 2001).

Based on this, we wanted to establish conditions in semi-intact cells to reproduce the *in vivo* targeting. The semi-intact procedure consists in treatment of cultured cells with low concentrations of digitonin, which permeabilises the plasma membrane, while leaving intracellular membranes intact. For my experiments I used the constructs 2 and 3 described in Figure 1. b5-RR is identical to b5-ER except for the last residues at the C-terminal, which are replaced with two arginines; this protein localises *in vivo* to the MOM and, as b5-ER, is able to be inserted into liposomes without assistance. For these experiments, b5-RR and b5-ER were purified as recombinant fusion proteins, isolated from bacteria transformed with the corresponding plasmids (Figure 2, A and B). HeLa cells were transfected for 24 hours with RFP-KDEL, which serves as ER marker. The day after the transfection, each recombinant protein, in the presence of buffer (KHM) alone or of cytosol (RRL) was incubated with semi-intact cells for 1 hour at 26°C (see Methods). The cells were then washed, fixed and analysed by immunofluorescence. The antibodies used were the anti-b5 (in green) and anti-Tom20 as MOM marker (in blue).

We found that b5-RR localised to the MOM both in KHM and RRL, as can be seen by the co-localisation of b5 with Tom20 (Figure 3A). This means that this protein does not need cytosolic factors to reach its final destination, since in buffer there was already 75% of colocalisation to the MOM; however, in RRL this percentage increased to almost 90% (Figure 3A), suggesting that factors in the RRL could facilitate targeting to the MOM. We often observed swollen mitochondria, with fragmentation of the mitochondrial network. This can be explained by the procedure of cell permeabilisation and maybe by the overloading of

protein to this organelle. Despite this altered morphology of mitochondria, what is important to mention is that this structure is easily distinguisable from the structure of the ER, given by RFP-KDEL marker.

When we looked at b5-ER's localisation, very surprisingly we found that in buffer it also localised to the MOM, in the same way as b5-RR. After quantification, we concluded that the target of b5-ER to MOM is as efficient as b5-RR, with a percentage of co-localisation of almost 80% (Fig. 4A). On the contrary, when b5-ER was incubated with RRL, the majority was localised to the ER, as quantified by the co-localisation with the marker RFP-KDEL (~65%, Figure 3B and 4B). This is particularly well observed in the inset of Figure 3B, where the nuclear envelope indicated by the arrow is a clear evidence of ER localisation. Despite the fact that there was still some b5-ER mislocalised to the MOM, this is significantly different from the b5-RR colocalisation to the MOM (Figure 3A and 4A). The same thing is true for the opposite situation: despite there was some b5-RR localised to the ER in RRL, the colocalisation of b5-ER to with the ER marker in RRL was significantly higher.

The presence of some b5-ER on the MOM is in agreement with studies on the location of the two endogenous isoforms: while the mitochondrial isoform is exclusively present at the MOM, a small proportion (about 5%) of the ER form is also at the MOM (D'Arrigo *et al.*, 1993). In addition, the localisation of b5-ER to the MOM assessed by Mander's coefficient may be an overestimate, due to incomplete resolution between the ER and the MOM. Indeed, the ER marker, RFP-KDEL, was also found to partially co-localise with TOM20 (approximately 30%, data not shown), although it is most likely that there is no mitochondrial localisation of this marker. The same reasoning probably explains the percentage of b5-RR estimated to be present on the ER by Mander's coefficient.

The results presented in Figs. 3 and 4 led to the conclusion that b5-ER needs cytosolic factors to reach its organelle and to avoid mislocalisation to the MOM; on the other hand, b5-RR reaches its specific organelle in both conditions, suggesting that the mitochondria is somehow the default destination of TA proteins.

2. The targeting of b5-ER to the ER is energy dependent.

Once we have established that, in semi-intact cells, b5-ER is faithfully targeted in presence of RRL, we then asked if this targeting was energy dependent. To address that, we prepared a RRL depleted of ATP via a hexokinase/glucose trap (see Methods).

As expected, for b5-RR we saw no difference in its localisation to the MOM with or without energy. As accessed by the Mander's coefficient quantification, the difference in localisation to the MOM between normal RRL and energy depleted RRL was not significant. Nevertheless, the MOM targeting was higher in both these two conditions when compared to buffer, suggesting that RRL could have a minor role in the targeting process.

Differently, b5-ER's localisation to ER was significantly decreased in the energy depletion condition. Indeed, whereas in RRL there was approximately 65% of b5-ER co-localising with RFP-KDEL, in energy depleted RRL this value is less than 40% (Figure 4B). Conversely, co-localisation of b5-ER with the MOM in this condition was significantly higher than in normal RRL, which means that at least some part of the protein is relocated to the MOM when the targeting to the ER is not possible.

Regarding b5-RR's localisation to the ER, we observed that the small population that was colocalising to the ER decreases when it was incubated with RRL depleted of energy, once again supporting the idea that energy-dependent chaperones are required to reach this organelle.

3. With energy depletion there is less insertion into membranes.

Parallel with the finding that the targeting of b5-ER to the ER is energy-dependent, we also observed that the intensity of b5-ER's cell-associated fluorescence was much lower in the condition of energy depletion compared to the condition with complete RRL (Figure 4C). This might have happened due to the fact that the protein, in the absence of cytosolic chaperones, remains aggregated, i.e., non-functional, in the cytosol. Despite the fact that a

part can be relocated to the mitochondria, as said before (Figure 4A), the rest that is not able to reach the ER is probably washed away at the end of the incubation period.

As shown in Fig. 4C, the cell-associated intensity of fluorescence of b5-RR was also decreased after incubation with energy-depleted RRL. In this case, the explanation is not so obvious since as we have observed previously, b5-RR does not require RRL to be targeted to the MOM. In the energy depletion condition, it is likely that some b5-RR is trapped with ATP-dependent chaperones and is thus unable to reach its target membrane.

4. b5-RR and b5-ER are correctly integrated into the lipid bilayer.

Once we assessed by immunofluorescence the localisation of b5 forms to the ER and/or to the MOM, we then wanted to prove that they were inserted and not simply associated with membranes. To distinguish between these two situations, we performed a carbonate extraction experiment. Upon incubation of b5-ER and b5-RR with semi-intact cells, the homogenates of cells were treated with sodium carbonate, which maintains protein-lipid interactions while disrupting protein-protein interactions (see Methods). After separation of carbonate-soluble and insoluble proteins, we observed that all b5 was found in the pellet fraction, after incubation both with buffer and with RRL (Figure 5). b5 behaved the same way as the integral protein calnexin, and differently from the soluble protein PDI, which was found only in the supernatant fraction. This was also true in the condition b5-ER in buffer, which means that even when b5-ER is mistargeted to the MOM, it is fully integrated and not merely attached to the membrane of this organelle. In conclusion, despite the condition or the target organelle, b5 is always inserted into the lipid bilayer efficiently as an integral membrane protein.

5. b5-ops, with an N-glycosylation site, behaves like the non-tagged protein, b5-ER.

To confirm the information obtained with immunofluorescence experiments with biochemical ones, we performed the same experiments using the recombinant protein b5-

ops (construct 4 in Figure 1). This construct contains at the C-terminus a tag derived from the N-terminal sequence of bovine opsin; this tag contains a site for N-glycosylation, which allows the detection of translocation of the protein's C-terminus into the ER lumen, where the glycosylation is carried out by the enzyme oligosaccharyl-transferase.

We first assessed the localisation of this protein by immunofluorescence, in two different experiments. The first one shows the co-localisation of b5-ops with the MOM marker, Mitochondrial Fission Factor (MFF). As can be seen in Figure 6A, in buffer b5-ops perfectly co-localised with MFF, meaning that it was localised to the MOM (upper panel). On the other hand, in RRL the protein showed an ER pattern and, indeed, there was almost no co-localisation with the MOM marker (bottom panel).

To further demonstrate the ER targeting, we repeated the experiment using an ER marker for comparison (Figure 6B). In this case, the day before the cells were transfected with extended-Synaptotagmin-1 (E-Syt1-GFP, construct 9 in Figure 1). As expected, in RRL we observed co-localisation between b5-ops and E-Syt1-GFP, indicating its targeting to the ER (Figure 6B, bottom panel). Yet, as occurred with the non-tagged b5-ER, some co-localisation with the MOM marker was also observed. Nevertheless, it is clear that in buffer, the co-localisation with Tom20 is almost complete (Figure 6B, upper panel). Thus, b5-ops requires cytosolic chaperones to reach the ER and, in the absence of cytosol, is mistargeted to the MOM, similarly to b5-ER.

To demonstrate the insertion into the ER biochemically, at the end of the incubation of b5-ops with semi-intact cells, the cells were lysed and analysed by SDS-PAGE and WB (Fig. 7 -see Methods). When the incubation was done with RRL, the western blot showed an additional band that migrated approximately 3 KDa higher than the one of b5 (~20 KDa - Fig. 7A). This band corresponds to the glycosylated b5, as demonstrated by its sensitivity to Peptide-N-glycosidase F (PNGase F- Fig. 7C), and it indicates that the C-terminus of b5-ops was translocated into the ER lumen, where the glycosylation machinery resides (Figure 7A). The quantification of the percentage of protein glycosylated revealed that it was >25% in RRL, and that this value was significantly different from the 4% obtained in buffer (Figure 7B). In this case, the efficiency of glycosylation reflects not only the quantity of b5 that

successfully reached the ER but also the capacity of the glycosylation machinery. It is possible that the amount of protein that we are adding to the cells is in excess compared to the saturation limit of the enzymes involved in this process. Moreover, the presence of an accessible, correctly-oriented glycosylation site, is an absolute requirement for an efficient glycosylation.

Since b5-ER relies on energy-dependent factors to be targeted to the ER, we investigated this requirement for b5-ops with our biochemical assay. b5-ops was incubated with semi-intact cells and with RRL depleted of energy, and was analysed by SDS-PAGE followed by Western Blot. In conditions without energy, b5 was no longer glycosylated (or very weekly), as expected from the immunofluorescence results (Figure 7D). In addition, under this condition, the band of non-glycosylated b5 is weaker, demonstrating once again that with energy depletion there is less association of b5 with membranes.

6. b5-ops import depends on time and cytosol concentration.

To better characterise the system with semi-intact cells that we have developed, we first asked ourselves how dependent is b5 on the cytosolic factors present in the RRL. For that, we incubated b5-ops with different fractions of RRL diluted in KHM. As seen in the graph A of Figure 8, when RRL was diluted 1:2, glycosylation was almost as efficient as in the case of complete RRL; however, with the dilutions 1:4, 1:6 and 1:8, the glycosylation decreased dramatically, reaching 0% when incubated only with KHM.

Then, we performed a time-course experiment, in which semi-intact cells incubated with b5-ops were collected at different time-points: 15, 30, 45 and 60 minutes (Figure 8B). The glycosylation of b5, in other words, the insertion of b5 into the ER, increased concomitantly with the incubation time, reaching its maximum after 1 hour of incubation with RRL. Finally, we were interested to know whether at the different time-points b5 had different localisations, for instance, if at early time-points was first wrongly inserted into the MOM and only after was relocated to the ER. This idea came from the work of Chen *et al.* (2014), who reported that a protein named ATAD1 (Msp1 in yeast), was able to extract mislocalised mitochondrial TA proteins, thus maintaining mitochondrial integrity. To do this, we used the

non-tagged protein, b5-ER, and we compared its localisation with markers of ER and of MOM, respectively RFP-KDEL and Tom20. After 5 and 15 minutes of incubation with RRL, b5-ER showed a dual localisation, both ER and MOM, as quantified by Mander's co-localisation coefficients (Figure 8C). At 30 minutes, the localisation to the MOM had reached a *plateau*, while the ER localisation continued to increase. Since the mitochondria-associated fluorescence never decreased, the results suggest that the MOM-localised fraction of b5-ER is not a precursor to the ER-targeted portion; rather, the results suggest that b5-ER is directly targeted to the ER in a relatively slow process.

7. TRC40 pathway plays a minor role in b5-ops targeting.

As described in the introduction, the main pathway for post-translation insertion of TA proteins into the ER is the TRC40 pathway (Stefanovic and Hegde, 2007). TRC40 is an ATPase that binds to hydrophobic TMDs of proteins and delivers them to the ER. Although it has been reported that cytochrome b5 does not need assistance for its insertion (Favaloro *et al.*, 2008; Brambillasca *et al.*, 2006), an interaction of b5 with TRC40 has also been demonstrated (Colombo *et al.*, 2009; Stefanovic and Hegde, 2007; Favaloro *et al.,* 2010).

We therefore wondered whether TRC40 had a role in the targeting of b5-ER. To study this pathway, we applied different approaches. First, we interfered with TRC40 taking advantage of the protein WRBcc, which acts as a decoy receptor. WRBcc corresponds to the coiled coil portion of WRB, and it is the binding site of TRC40-TA complex (Vilardi *et al.*, 2011). By adding WRBcc to our semi-intact system, it competes with resident WRB at the ER membrane, thus blocking the delivery of TRC40-TA complexes to its receptor. WRBcc wild-type (WRBcc WT) or a mutant, inactive, version (WRBcc MUT) were expressed in bacteria and purified (see Methods and Figure 9A), incubated with b5-ops and RRL at a concentration of 5 µM and applied to semi-intact cells. The western blot revealed a weaker glycosylated band of b5 after incubation with WRBcc WT (Figure 9B). Quantification of the blots revealed that WRBcc caused a modest, but significant decrease of b5's insertion into the ER, when compared to WRBcc MUT (graph in Fig. 9B). As positive control for the effect of WRBcc, we used another construct, syb2-ops (construct 7 in Figure 1A). This construct corresponds to

cytochrome b5 with the TMD of synaptobrevin-2, which is a *bona fide* substrate of TRC40. Since the TMD of this protein is highly hydrophobic, it was impossible to obtain the recombinant protein from bacteria in the absence of detergent, and so we performed this experiments doing *in vitro* translation of both syb2-ops and b5-ops TA proteins. We found that differently from b5, whose glycosylation was only slightly affected by the addition of WRBcc, syb2's glycosylation was almost completely abolished (Figure 9C). Thus, while a great majority of b5 can still reach the ER when TRC40 is blocked, syb-2 certainly depends on this chaperone for its insertion also in semi-intact cells, in agreement with previous results in cell-free systems (Colombo *et al.*, 2016).

To further investigate the role of WRB/TRC40, we repeated the same experiments by siRNA-mediated silencing of WRB. The silencing was done for 48 hours with two different siRNA's and the efficiency of silencing achieved was always high (Figure 10A). We first used scramble or siRNA transfected HeLa cells to investigate the localisation of recombinant b5-ops by immunofluorescence. As seen in the Figure 10B, in both cases b5-ops showed an ER-like structure, but, when WRB was silenced, the co-localisation of b5-ops with the MOM marker Tom20 was more evident (yellow in the merge image, bottom panel). Notwithstanding the values of co-localisation with mitochondria obtained after quantification were relatively similar (43% in scramble and 49% in silenced cells), from a statistical point of view this difference was significant (data not shown).

The glycosylation of recombinant b5-ops in cells silenced for WRB (Figure 11C) was decreased by approximately 25%, in agreement with the results obtained with the WRBcc. This difference was again significant, when compared to the values of glycosylation obtained with scramble transfected cells. Once more, we performed *in vitro* translation to compare both b5-ops and syb2-ops. While b5's insertion into the ER was weakly affected after silencing of WRB, syb-2 showed again a strong dependence on the TRC40 pathway, since its glycosylation was reduced by 60% (Figure 11D). The differences in syb2's glycosylation between WRBcc addition and WRB knockdown, i.e., the less dramatic effect obtained in this last approach could be explained by the fact that, upon silencing, there is still some WRB available, which can perhaps be enough for the functioning of the TRC40 pathway. Hence,

the TRC40 pathway would be impaired, but not completely abolished; on the contrary, when the receptor is blocked by the WRBcc, the formation of complexes of TRC40-TA that cannot be delivered to the ER membrane would limit its insertion to a greater extent.

An alternative way to investigate the involvement of TRC40 was by depleting TRC40 from the RRL. An immunodepletion with anti-TRC40 antibodies and one with an unrelated IgG (mock depletion) were done in parallel. The depletion was checked by western blotting and, as shown in Figure 11A, the RRL was efficiently depleted. The TRC40 depleted lysate was then incubated with the recombinant b5-ops in semi-intact cells and the glycosylation was checked by western blotting. As shown in Fig. 11B and C, TRC40 depletion, like WRBcc and WRB silencing, had a modest effect on b5-ops glycosylation (~20% inhibition), although the difference with the control did not reach statistical significance.

Finally, the last experiment that was done to characterise the role of TRC40, although indirectly, was carried out by investigating the role of the chaperone SGTA in the targeting of b5 to the ER. As explained before in the introduction, SGTA is a chaperone that shields TA proteins from the aqueous cytosol, by recognising and binding to the hydrophobic exposed regions. It was demonstrated that SGTA can act as a bridge, by connecting substrates from the ribosomal exit to the Bag6 complex, the pre-targeting complex of TRC40 pathway. Furthermore, a recent structure-based study of the Bag6 complex suggests that SGTA can also directly hand off TA proteins to TRC40 (Roberts *et al.*, 2015). If SGTA does so for cytochrome b5, after the knockdown of this chaperone, we would expect to see less ER targeting and perhaps an increased targeting to the MOM.

Since SGTA is a cytosolic chaperone, we could not apply our import assay with semi-intact cells by adding RRL, which contains SGTA. So, to perform this experiment we applied a microinjection protocol in cells silenced for SGTA (Fig. 12A). Microinjection can be considered an acute transient transfection and, in this case, consisted in the injection of the recombinant b5-ER into the cytoplasm of cultured cells, followed by fixation after 15 minutes (Figure 12B). By immunofluorescence analysis we saw no difference in b5-ER localisation in either 48 hours of scramble or SGTA siRNA transfected cells (Figure 12C). In both cases, b5-ER displayed an ER-like structure and, indeed, the difference in the co-localisation with the

MOM between the two conditions was not significant (Figure 12D). Thus, b5-ER reaches the ER by one or more pathways not involving SGTA.

In conclusion, with these different approaches we concluded that the involvement of the TRC40 pathway in the delivery of cytochrome b5 to the ER membrane is minor. Most likely, b5 can take advantage of this chaperone if available; but, if not, b5 can use one or more additional pathways and still reach the ER.

8. The SND mediated pathway plays a minor role in b5-ops targeting.

Recently, an SRP-independent targeting system was discovered in yeast and termed SND system (Aviram *et al.*, 2016). This novel pathway involves the cytosolic component SND1, which binds to ribosomes and, subsequently, to its receptor in the ER membrane (comprising SND2 and SND3). To understand if the SND pathway may be involved in TA protein targeting, we silenced human SND2, the only mammalian orthologue described so far (Aviram *et al.*, 2016). The silencing was done with four different siRNA's for 72 hours and it was checked by western blotting (Figure 13A, upper panel). Then, after incubation of b5-ops with RRL in semi-intact cells, the % of glycosylation was analysed (Figure 11A, bottom panel). Since the results showed a very similar extent of glycosylation in all siRNA's tested (Figure 12A), we continue the experiments using only the SND2 siRNA#3.

It was suggested that the three pathways, SRP, SND and TRC40, work in parallel to facilitate the ER targeting of proteins bearing TMDs in all possible relative positions (Aviram *et al.*, 2016). Following this theory, we wondered what would happen to cytochrome b5 if both TRC40 and SND pathways were blocked. We therefore performed a double knockdown of SND2 and WRB. The silencing was done for 72 hours for SND2 and for 48 hours for WRB (Figure 12B). Confirming our previous observations, the individual knockdown of WRB decreased the percentage of b5's glycosylation by ~25%, while SND2 silencing had a milder effect (~15% inhibition). When both pathways were blocked, the effect was, at most, additive (graph of Figure 12B). Thus, despite the fact that these two pathways may play a role in b5's targeting, there are for sure other(s) pathway(s) involved, as more than 60% of the b5 protein is still able to be inserted into the ER in the absence of both of them. In

addition, since the effect obtained with the double knockdown tends to be additive, this suggests that these two pathways are independent.

9. No evidence for an involvement of heat shock proteins.

Another protein implicated in the targeting of b5 in vitro is Hsc70 (Abell *et al.*, 2007; Rabu *et al.*, 2008; Rabu *et al.*, 2009). To test its potential role, we treated the RRL with a series of chemical inhibitors: MAL2-11B, DMT002220 and DMT003024 (Fewell *at al.*, 2004). All of them inhibit the Hsp40-stimulated Hsp70 ATPase activity. We also evaluated the involvement of another chaperone, Hsp90, by treating cells with the antibiotic geldanamycin. Geldanamycin blocks the action of Hsp90, by binding to the unusual ADP/ATP-binding pocket of the protein.

After RRL treatment with the described drugs, individually, the cells were permeabilised and then incubated with the recombinant b5-ops in RRL (see Methods). None of the treatments succeeded in blocking the targeting of b5 to the ER. An example for MAL2-11B, a molecule with improved chemical properties derived from MAL3-101 (Figure 14A) is shown in Figure 14B. The effect was compared with DMSO treatment, the solvent in which MAL2-11B was dissolved, but in some experiments a molecule with no Hsp70 ATPase activity inhibition, MAL3-51, was also used as control (data not shown). The quantification of b5's glycosylation after Hsp70 inhibition was minor (<10%) and statistically non-significant when compared to DMSO (Figure 14B). The same was true for all the drugs tested.

10. ES I treatment affects b5, but not syb2, insertion.

The translocation of preprocecropin A, a short secretory protein, into ER derived microsomes is inhibited by the small molecule inhibitor eeyarestatin I (ES I) (Cross *et al.*, 2009; Johnson *et al.*, 2012). ES I is a potent inhibitor of ERAD, targets the p97-associated and ataxin-3- dependent deubiquinating processes, and inhibits Sec61-mediated protein translocation at the ER (chemical structure in Figure 14C). Based on this, we decided to check the effect of this drug in b5's targeting. Initially, we treated the RRL with 10 or 100 μM of ES

I, for 1 hour on ice; then, the recombinant b5-ops was added and incubated with semi-intact cells for 1 hour at 26 °C. We observed that the glycosylation of b5 was severely affected (Figure 14D). Even at 10 µM, ES I caused a 50% reduction of glycosylation (graph of Figure 14D), an effect that was stronger than that obtained with any of the other treatments. We repeated the experiment by doing *in vitro* translation of b5-ops and syb2-ops and by treating the translation mixtures at the end with ES I. Surprisingly, we found that the dramatic effect of ES I on b5's import, was not reproduced for syb2, which was glycosylated even more efficiently than under control conditions. This observation excluded the possibility that the drug was somehow interfering with the glycosylation machinery and not with the ER insertion.

Given the substrate-specific effect of ES I, we were interested to know whether this effect was shared with other spontaneously inserted TA proteins, e.g., PTP1B. Since the glycosylated PTP1B migrates very close to the non-glycosylated portion, the analysis of the glycosylation of this TA protein was done with concanavalin beads (see Methods). In this way we could compare b5, PTP1B and syb2 after treatment with ES I. As it can be seen in Figure 15A, the glycosylated band (bound fraction) of both b5 and PTP1B was weaker after treatment with ES I, while the glycosylated band of syb2 remained unaltered compared with the bound fraction of control lysates. The quantification (Figure 15A on the right) revealed that b5 and PTP1B were affected almost in the same way, with an inhibition in the glycosylation varying from 35 to 45%. In contrast, syb2's glycosylation was higher compared to control DMSO.

An analogue of ES I with a related structure, ES_{35} (Figure 14C), which is biologically inactive (Cross *et al.*, 2009), was used to decipher if the action of ES I on TA protein insertion correlates to its known biological activity. The effect of ES_{35} did not differ from the one of the vector (DMSO) and, as expected, differed from the one with ES I (Figure 15B). What is important to note is that, in all cases in which we analised the effect of ES I, the inhibition observed was not due to an increase of the percentage of non-glycosylated band (that we could expect since ES I is an ERAD inhibitor), but to a decrease in the glycosylated band (i.e., in the inserted protein).

As said before, ES I inhibits p97-associated deubiquination. Misfolded proteins are ubiquitinated and extracted from the membranes upon ATP hydrolysis of p97 and subsequently targeted to the proteasome for degradation. To explore if the effects of ES I where mediated through p97, we selected a potent and specific inhibitor of this ATPase, DBeQ (N^2,N^4-dibenzylquinazoline-2,4-diamine). DBeQ inhibits the degradation of ubiquitinated proteins, the ERAD pathway, and autophagosome maturation (Chapman *et al.*, 2015). Under the same conditions, the RRL was treated with this drug and the effect on b5-ops glycosylation was analysed. As easily observed by the western blot and the respective quantification (Figure 15C), the glycosylation of b5-ops remained unaltered. Thus, the effects of ES I in reducing the integration of b5-ops in the ER are not mediated by p97 ATPase inhibition.

11. Sec61 knockdown affects b5, but not syb2, insertion.

Lastly, since it is known ES I inhibits Sec61-mediated protein translocation at the ER, we decided to test the unlikely role of Sec61 translocon in the post-translational insertion of b5 into the ER. We silenced the α subunit of Sec61 with a double transfection for 72 hours and we verified that only the siRNA#2 was efficient (Figure 16A). First, we used Sec61 silenced cells to check the glycosylation of recombinant b5-ops (Figure 16B). We found a decrease in b5's insertion of approximately 25% (close statistical significance, $p = 0,052$). When the Sec61 knockdown was examined in *in vitro* translated b5-ops and syb2-ops proteins, we discovered that its effect was specific for b5 and not for syb2, which was almost not affected (Figure 16C). This goes in line with the results obtained after treatment with ES I and suggest that ES I affects b5's insertion by inhibiting the Sec61 translocon. Finally, we performed an experiment doing a double knockdown of Sec61 and WRB. We found that the inhibition of b5's glycosylation is the same whether Sec61 and WRB are silenced individually or together (Figure 16D). This might mean that they act in the same pathway, i.e., that TRC40 could deliver b5 to the ER membrane via Sec61 translocon. Alternatively, compensating pathways are activated in the silenced cells (see Discussion).
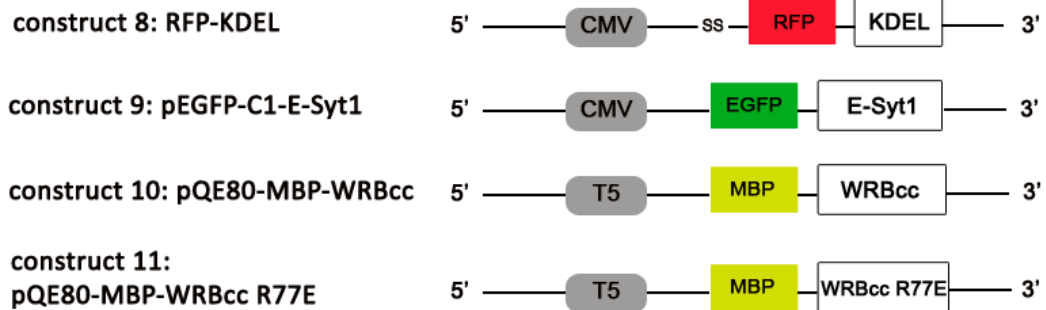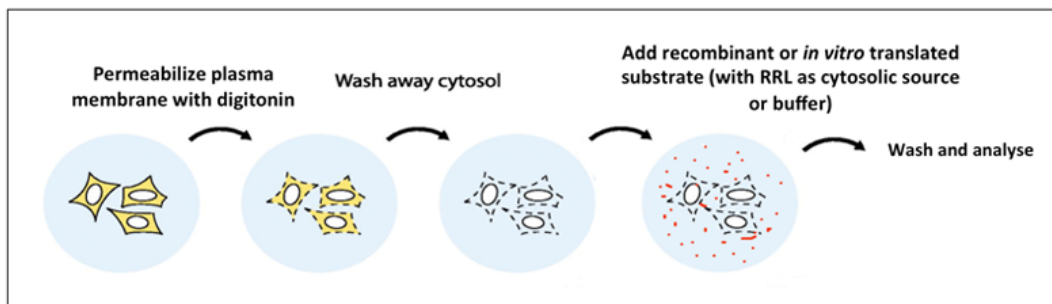
6. Figures and legends
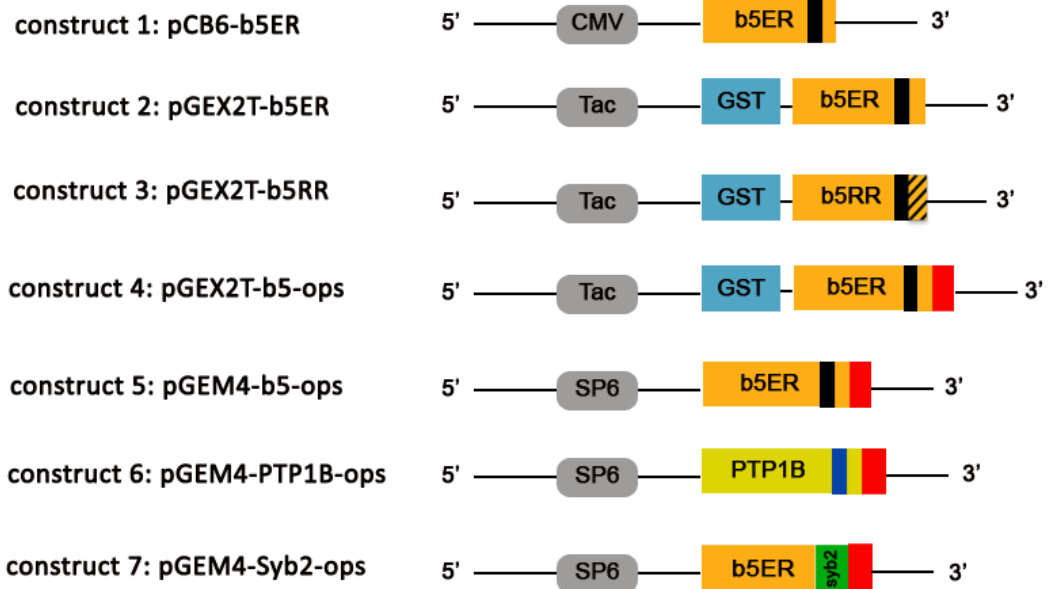
Figure 1: Constructs and protocol used for import assays.

A) Schematic representation of the constructs used in this work. The grey box shows the promoter of each plasmid and the blue box in constructs 2,3 and 4 the coding region for GST. The black rectangle represents the TMD of cyt b5, which is the same in constructs 1 to 5, and the red rectangle represents the opsin tag containing a N-glycosylation site. Construct 3, b5-RR, differs to b5-ER only in the last residues at the C-terminal (depicted as a dashed rectangle). Construct 7, Syb2-ops, contains the sequence of b5 but the TMD of synaptobrevin 2 (green rectangle). Construct 6, which codes for PTP1B (yellow, with TMD in blue), also contains the C-terminal opsin epitope. Constructs 8 and 9, under the CMV promoter, were used as ER markers and were obtained from Erik Snapp and Pietro De Camilli, respectively. Construct 1 (obtained from Fabio Vilardi and used to block the TRC40 pathway), and construct 11 (corresponding to a mutant version of construct 10, from Stephen High) code for Maltose Binding Protein (light green boxes) fusion proteins and, like the pGEX constructs, are under an IPTG-inducible promoter.

B) Scheme of the protocol used for import assays. Adherent cells were permeabilised with digitonin, 5 minutes at 26°C, to empty their cytosolic content; then, the *in vitro* translated or the recombinant protein was incubated with the cells together with rabbit reticulocyte lysate (RRL) or buffer (KHM), for 1 h at 26°C. At the end, the cells were washed and analysed by western blot or immunofluorescence.
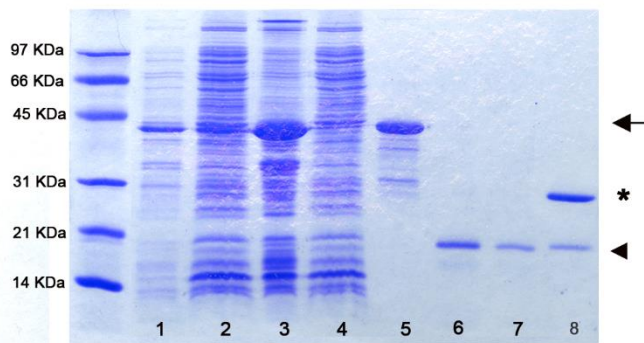
A

b5-ER
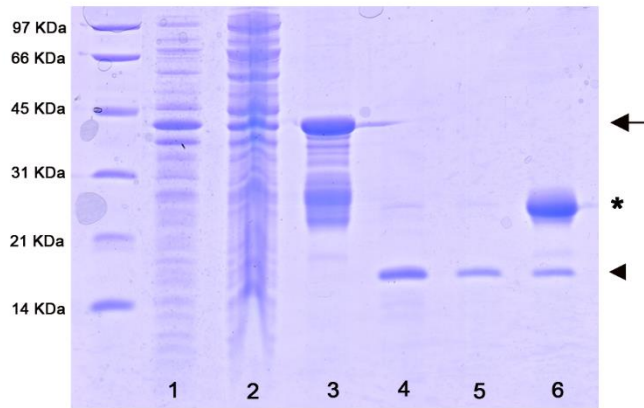
1- total lysate
2- unbound to GSB4 resin
3- bound to GSB4 resin
4- eluate obtained after thrombin digestion
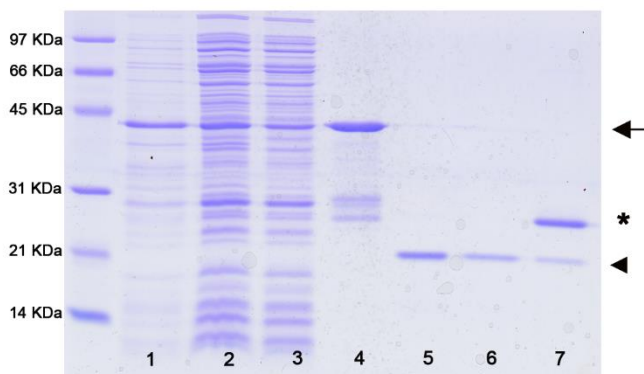5- wash
6- resin after thrombin digestion

B

b5-RR

1- total lysate
2- soluble fraction
3- insoluble fraction
4- unbound to GSB4 resin
5- bound to GSB4 resin
6- eluate obtained after thrombin digestion
7- wash
8- resin after thrombin digestion

C

b5-ops

1- total lysate
2- soluble fraction
3- unbound to GSB4 resin
4- bound to GSB4 resin
5- eluate after thrombin digestion
6- wash
7- resin after thrombin digestion

Figure 2: Purification of recombinant proteins.

CBB-stained SDS-polyacrylamide gels showing the purification of b5-ER **(A)**, b5-RR **(B)** and b5-ops **(C)** proteins. All three were expressed in BL21 bacteria as GST fusion proteins. After lysis of the bacteria, the soluble fraction was incubated on a rotating wheel with GSH-Sepharose 4B beads (GSB4 resin). Between the sequence of the GST and the sequence of b5 there is a cleavage site for thrombin, which results in the release of b5 protein by the enzyme (see Methods). The samples containing the soluble, insoluble and unbound fractions, were loaded in 12-fold excess over the total lysate; the lanes of bound to resin, eluate, wash and resin after thrombin were loaded in 16-fold excess over the soluble fraction, and are thus comparable each other. Note that most of the fusion protein is insoluble, but sufficient amounts can be purified from the soluble fraction. All of the soluble fusion protein binds to the resin and can be eluted by thrombin treatment. In each panel, the arrow, asterisk, and arrowhead indicate the fusion protein, cleaved GST, and cleaved recombinant b5, respectively. Note that the cleaved GST remains associated with the resin.

A

B
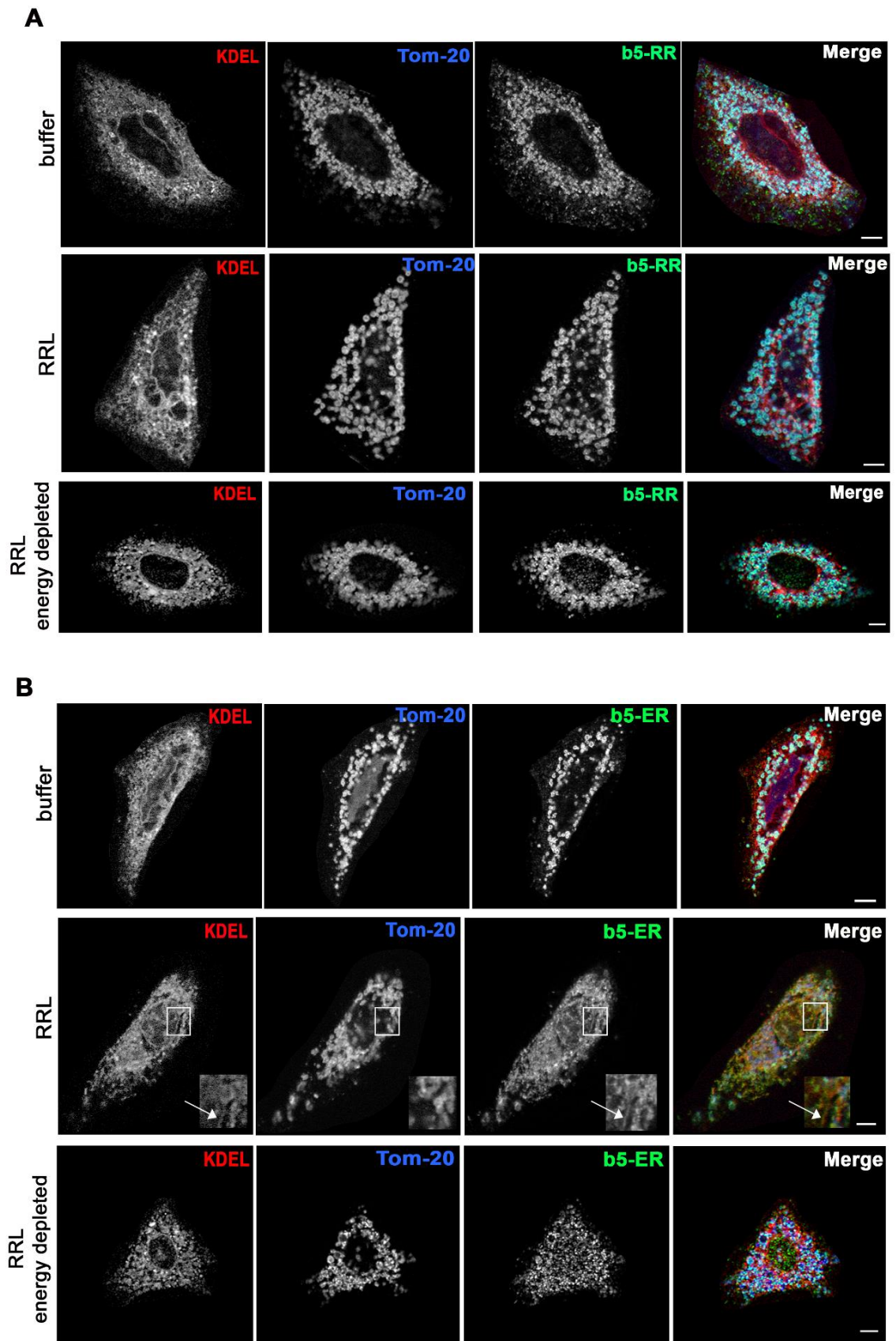
Figure 3: Localisation of recombinant b5 forms in semi-intact cells.

The cells were transfected with RFP-KDEL (ER marker, in red) and, 24 hours thereafter, permeabilised with digitonin and incubated with recombinant b5-RR **(A)** or b5-ER **(B)**, either in buffer (KHM), RRL, or energy-depleted RRL, as indicated. The cells were processed for immunofluorescence with anti-b5 antibody (green) and anti-Tom20 (MOM marker, in blue). In panel B, the arrows indicate the nuclear envelope, diagnostic for ER localisation (inset: 2x magnification). The cumulative results of quantification of cells like the one of this figure are shown in Figure 5. Single sections acquired with the LSM Meta microscope are shown. Scale bar = 5 μm.

A

Localisation to MOM

B

ER localisation

C

Fluorescence intensity
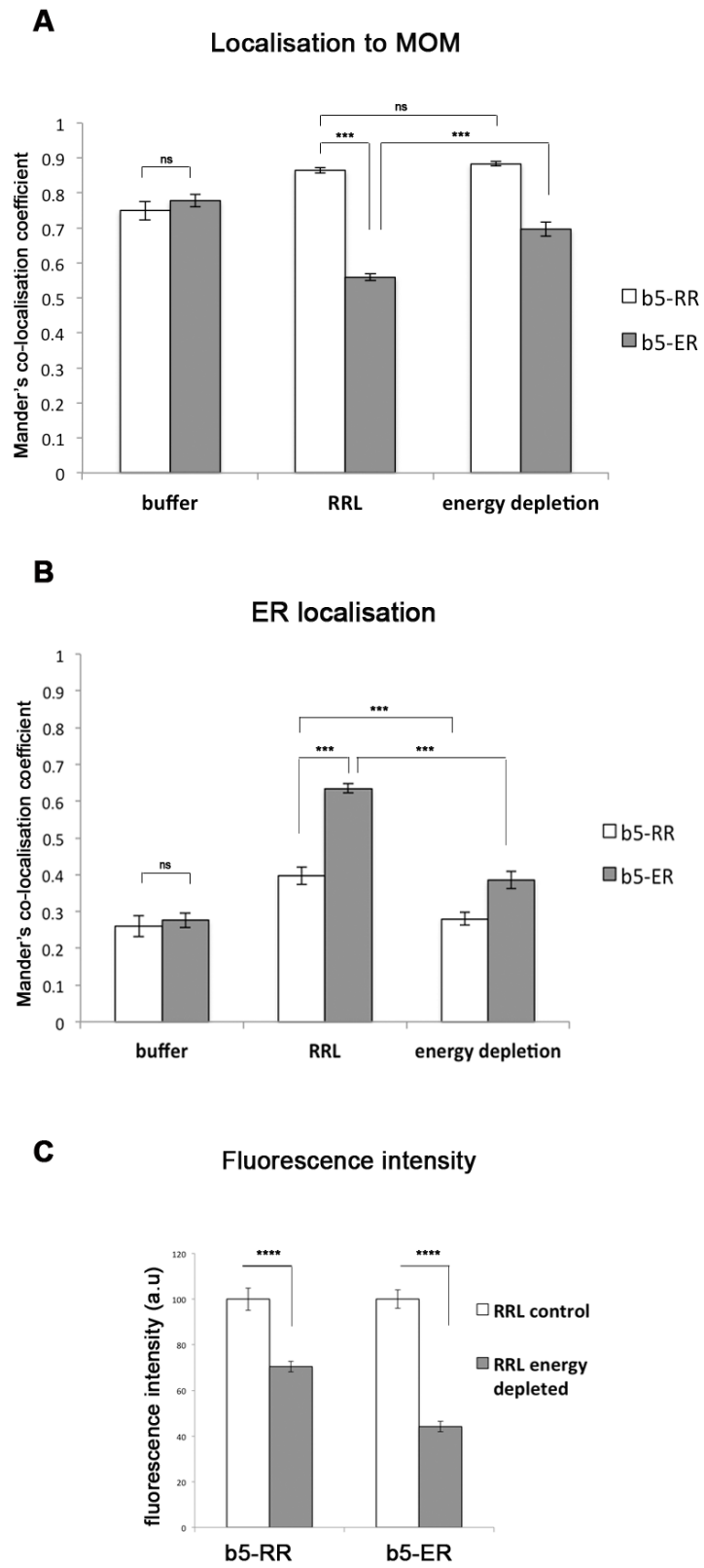
Figure 4: Quantitative analysis of the localisation of b5-ER and b5-RR under three different conditions (buffer, RRL and RRL with energy depletion).

A) Localisation of b5-RR (white) and b5-ER (grey) to the MOM. This corresponds to the percentage of b5 protein that co-localises with the MOM marker, Tom20. **B)** Localisation of b5-RR (white) and b5-ER (grey) to the ER. This corresponds to the percentage of b5 protein that co-localises with the ER marker, RFP-KDEL. In **(A)** and **(B)**, co-localisation of cytochrome b5 with mitochondria or ER markers was quantified by Mander's co-localisation coefficients (see Methods). Data presented are means ± SEM (number of cells analysed: b5-RR in KHM, 22; b5-RR in RRL, 57; b5-RR in energy depleted RRL, 51; b5-ER in KHM, 33; b5-ER in RRL, 110; b5-ER in energy depleted RRL, 64). Statistical significance was assessed by one-way Anova followed by Bonferroni's post-test.

C) Total cell-associated fluorescence intensity (in arbitrary units) of b5-RR and b5-ER after incubation in the presence of RRL and energy depleted RRL. Data presented are means ± SEM. The significance for plus vs minus energy for each construct was assessed by Student's t test (number of cells analysed: b5-RR in RRL, 22; b5-RR in energy depleted RRL, 48; b5-ER in RRL, 60; b5-ER in energy depleted RRL, 80).
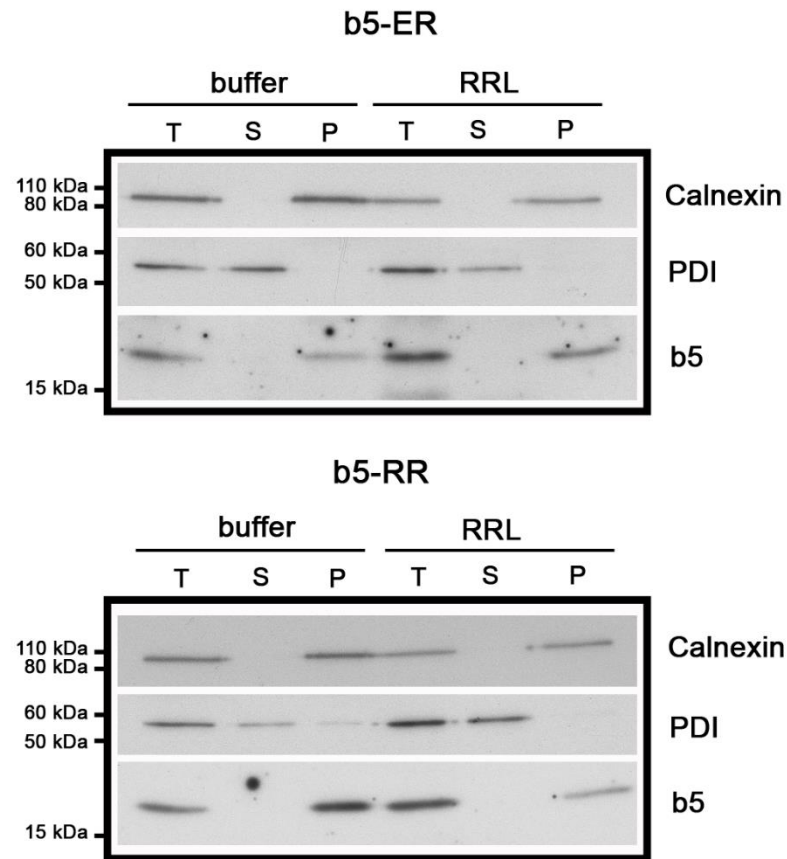
Figure 5: Analysis of b5 integration into the lipid bilayer by the carbonate extraction method.

After incubation of b5-ER (top) or b5-RR (bottom) with semi-intact cells, as in Figs 3-5, cells were homogenised and treated with Na_2CO_3 as described in the Methods. Equal aliquots of total sample (T), supernatants (S), and pellets (P) were analysed by western blot. Resistance to extraction of b5-ER (upper image) and b5-RR (bottom image) demonstrates that b5 is integrated into membranes after incubation either in buffer or in RRL. Calnexin and PDI were analysed as controls for an integral membrane protein and a soluble lumenal ER protein, respectively.

A
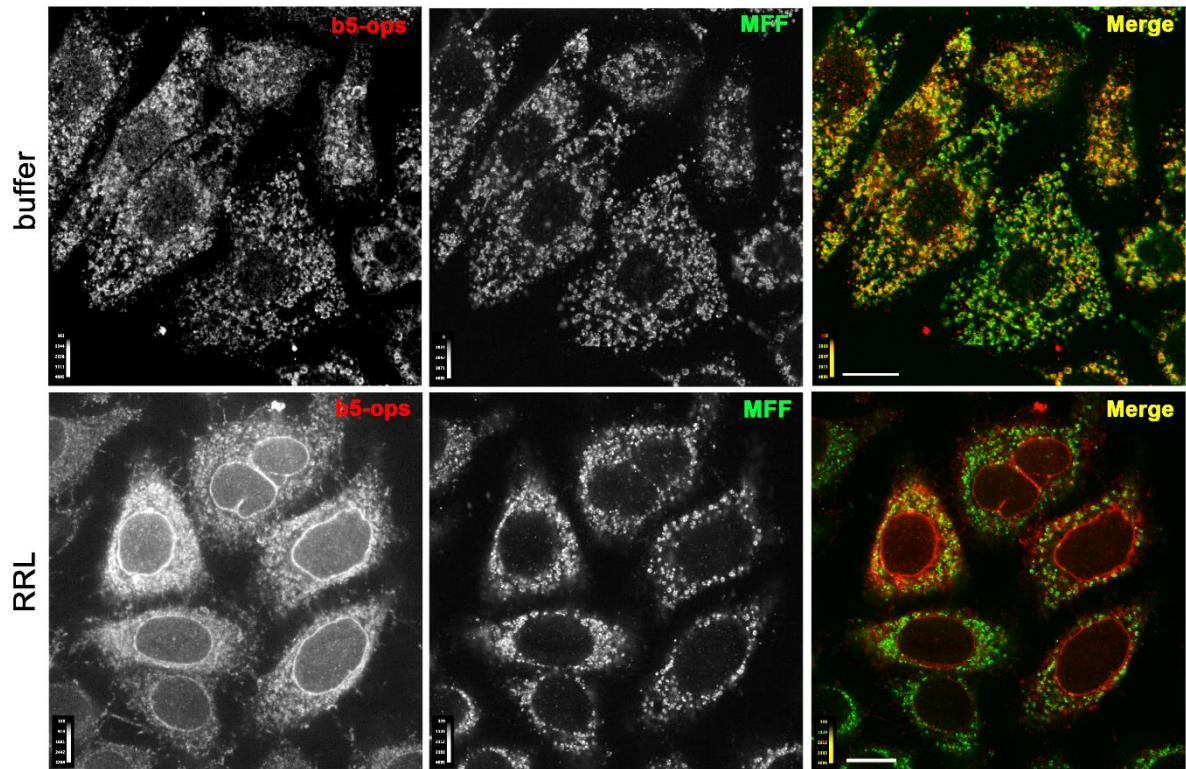
B
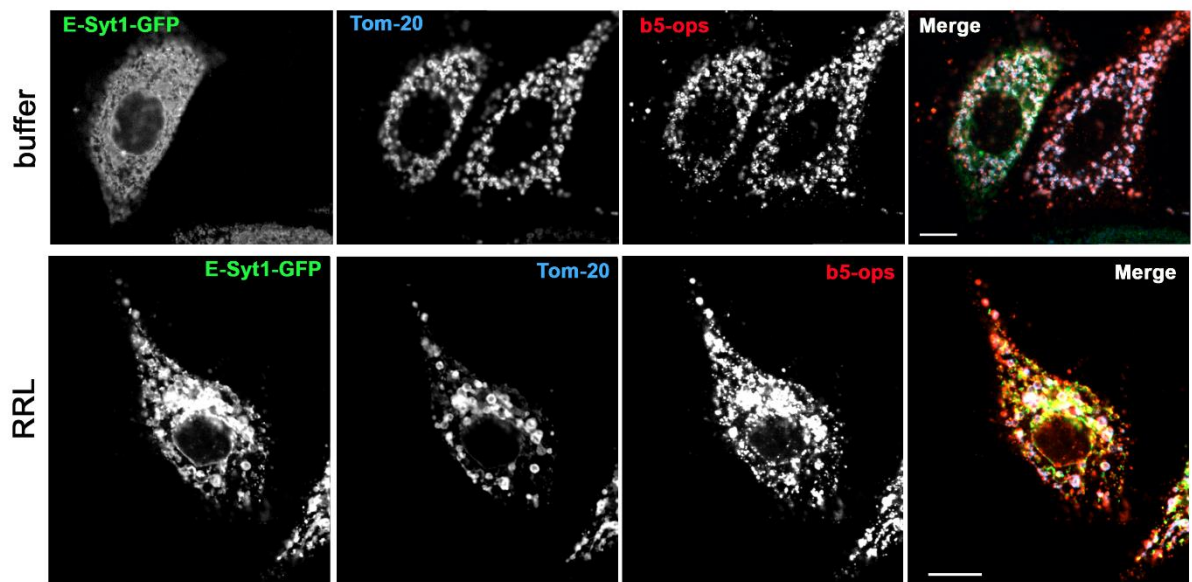
Figure 6: Localisation of b5 -ops in semi-intact cells.

Recombinant b5-ops was incubated with semi-intact cells in buffer (KHM) or RRL, as indicated. Panel **A** illustrates the lack of co-localisation of the construct with the MOM marker and its marked staining of the nuclear envelope, indicative of ER localisation. Panel **B** confirms the ER localisation of the construct by comparison with a transfected ER maker (extended Synaptotagmin 1). In this case, the cells were transfected, and semi-permeabilised 24 h thereafter. A: cells were stained with anti-opsin mAb and anti-MFF (MOM marker) polyclonal antibodies, as indicated. B: cells were stained with anti-Tom-20 mAb and anti-b5 polyclonal antibodies, as indicated. In A and B, single sections were acquired with a Confocal Spinning Disk LCI Ultraview system. Scale bar = 10 µm.
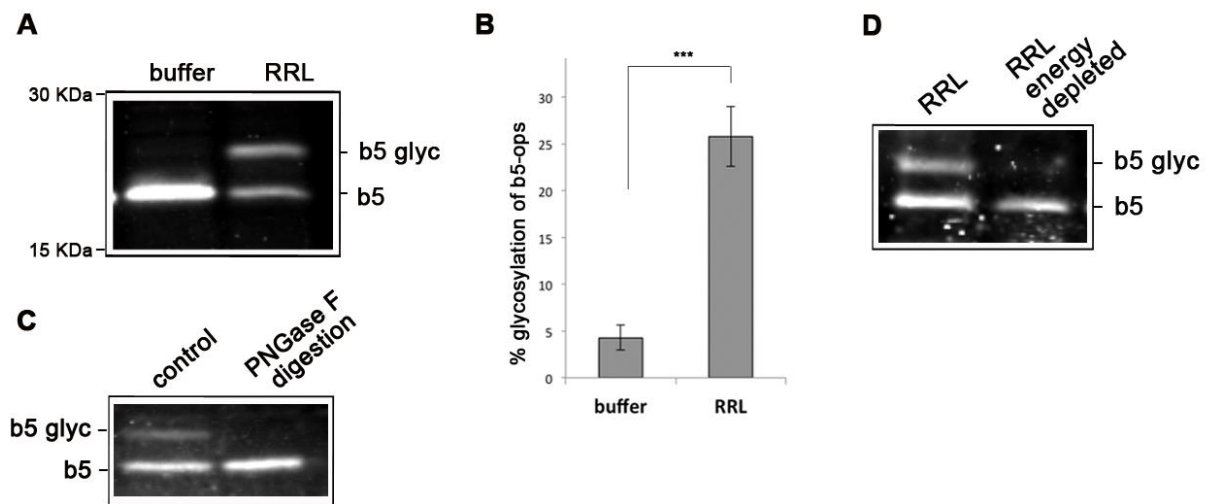
Figure 7: Biochemical analysis of b5-ops.

A) Representative western blot of b5-ops after incubation with semi-intact cells in the presence of buffer or RRL. Equal amounts of cell lysates were loaded and the transferred proteins were immunoprobed with anti-opsin mAb. The band corresponding to the glycosylated b5 is indicated (b5 glyc).

B) Quantification of the percentage of glycosylation of b5-ops in buffer and RRL (related to Figure A). Data are presented as mean ± SEM (n=5).

C) The cells were subjected to immunoprecipitation with anti-opsin antibody; the immunoprecipitates were divided in two, and one part was digested with PNGase F (see Methods) before SDS-PAGE/WB analysis as indicated.

D) Representative western blot of b5-ops in semi-intact cells incubated with RRL or RRL depleted of energy. Equal amounts of cell lysates were loaded and the transferred proteins were immunoprobed with anti-opsin mAb. The band corresponding to the glycosylated b5 is indicated (b5 glyc).
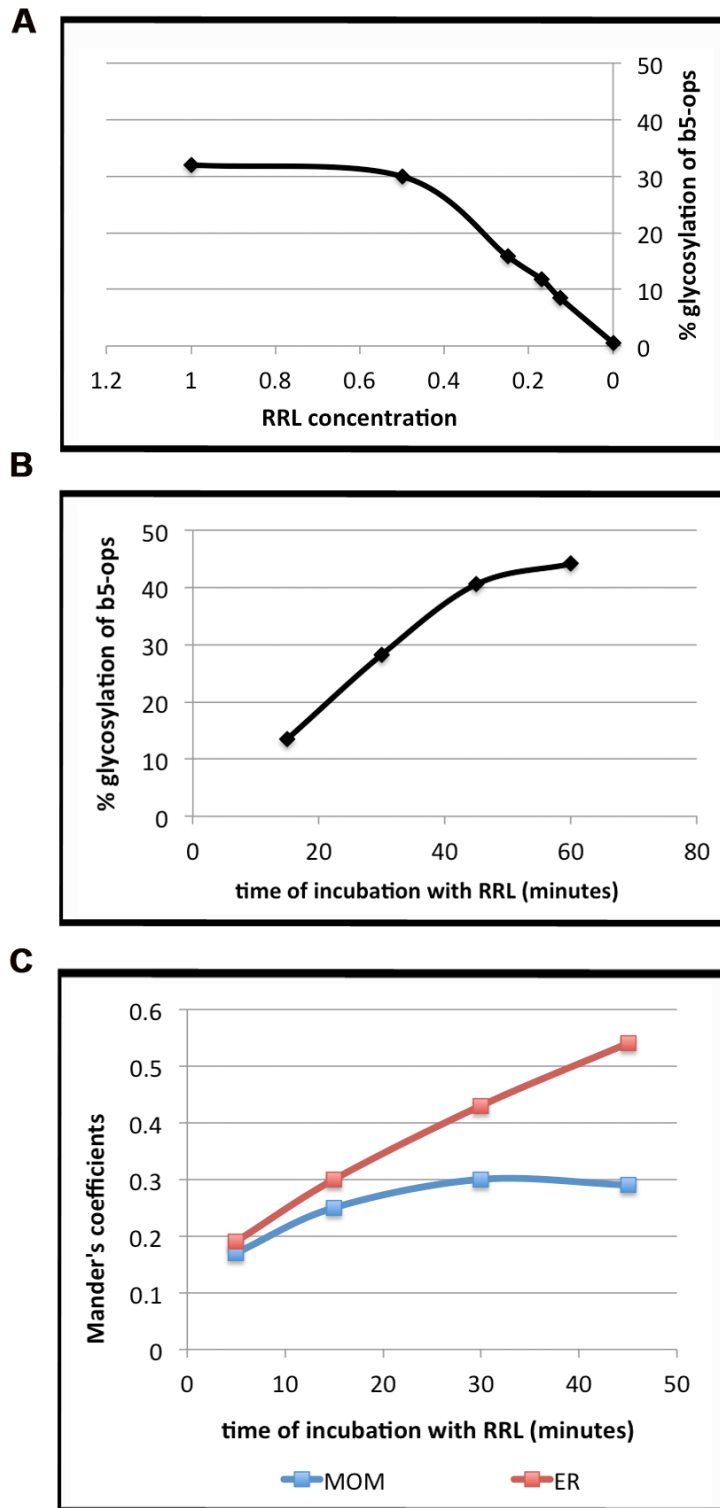
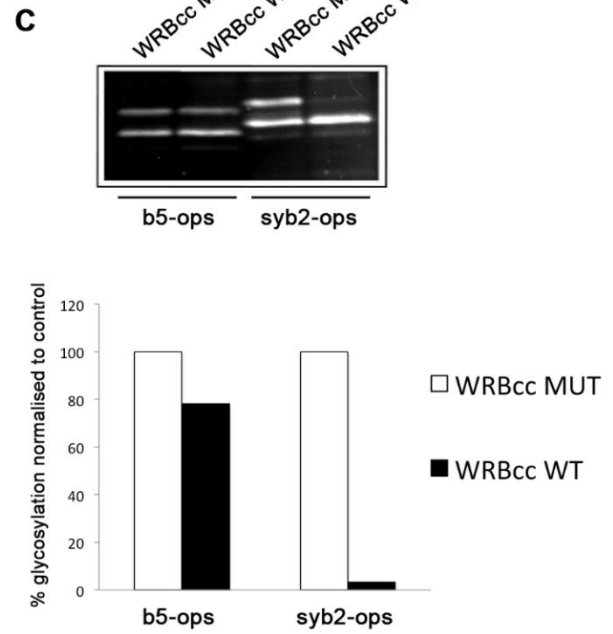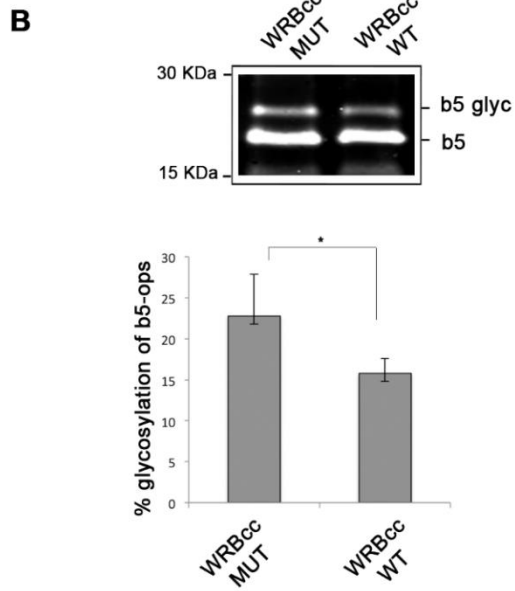## Figure 8: Time course and cytosol-dependence of b5-ops import.

A) The percentage of glycosylation of b5 depends on the RRL concentration. Cells were incubated for 1h with RRL under standard conditions, and with RRL diluted with KHM as indicated.

B) The percentage of glycosylation of b5 increases concomitantly with the time of incubation of the protein with RRL at 26°C. Cells were lysed at the indicated time points and analysed by SDS-PAGE/WB.

C) Time course of localisation of b5-ER in RRL. Cells were fixed at the indicated time points and analysed by immunofluorescence. ER localisation was accessed with RFP-KDEL and MOM localisation with Tom20.

A

66 KDa
45 KDa → MBP-WRBcc

1 2 3 4 5 6 7 8

1- total lysate
2- soluble fraction
3- insoluble fraction
4- soluble fraction diluted
5- unbound to amylose resin
6- bound to amylose resin
7- eluted with maltose
8- resin after maltose elution

MBP-WRBcc   MBP-WRBcc MUT

45 KDa → MBP

14 KDa → WRBcc

B

WRBcc MUT   WRBcc WT

30 KDa
— b5 glyc
— b5
15 KDa

C

WRBcc MUT   WRBcc WT   WRBcc MUT   WRBcc WT

b5-ops   syb2-ops
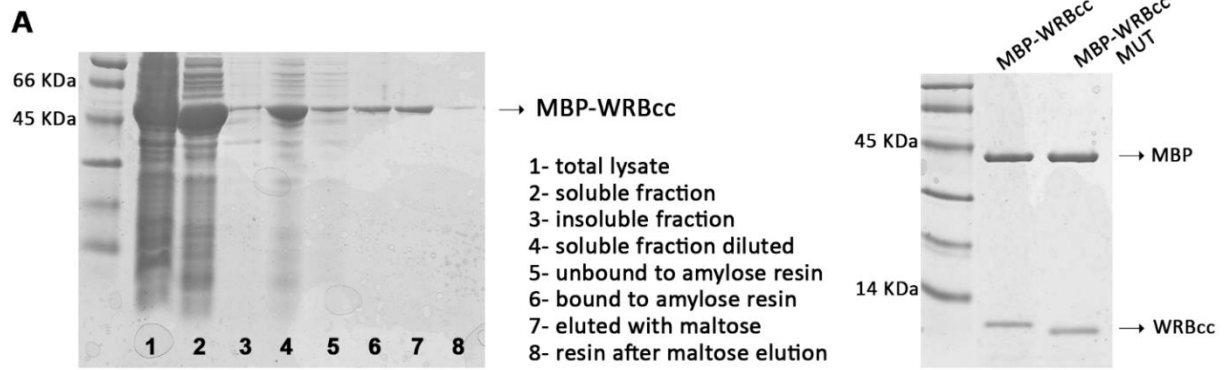
☐ WRBcc MUT
■ WRBcc WT

Figure 9: Effect of WRBcc on ER insertion of b5 -ops and Syb2-ops.

A) CBB-stained gel showing the purification of MBP-WRBcc. MBP-WRBcc and MBP-WRBcc MUT were purified from the soluble fraction of an IPTG induced BL21 sonicate and incubated with amylose resin (gel on the left). Most of the protein is soluble (lanes 1-3), but, under our conditions, not all of the fusion protein binds to the amylose resin (lanes 5,6). Nearly all the bound protein can be eluted with maltose (lanes 6,7). WRBcc MUT behaved in the same way as the wild-type protein (not shown). In both MBP-WRBcc wild-type and MUT, incubation with TEV protease allows the cleavage of WRBcc (MW=9 KDa) from MBP (MW=42.5 KDa) (gel on the right). Volumes loaded are comparable in all lanes.

B) Representative western blot of recombinant b5 -ops incubated with semi-intact cells in the presence of RRL pre-treated with 5 µM WRBcc MUT or WRBcc WT. Equal amounts of cell lysate were loaded and the transferred proteins were immunoprobed with anti-opsin mAb. The band corresponding to the glycosylated b5-ops is indicated (b5 glyc). Quantification of the percentage of glycosylation of b5-ops is shown in the graph below. Data are presented as means ± SEM (n= 3).

C) Representative western blot of *in vitro* translated b5-ops and syb2-ops incubated with semi-intact cells in the presence of RRL treated with 5 µM WRBcc MUT or WRBcc WT. Equal amounts of cell lysates were loaded, and the transferred proteins were immunoprobed with anti-opsin mAb. Quantification of the percentage of glycosylation of b5-ops and syb2-ops is shown in the graph below.
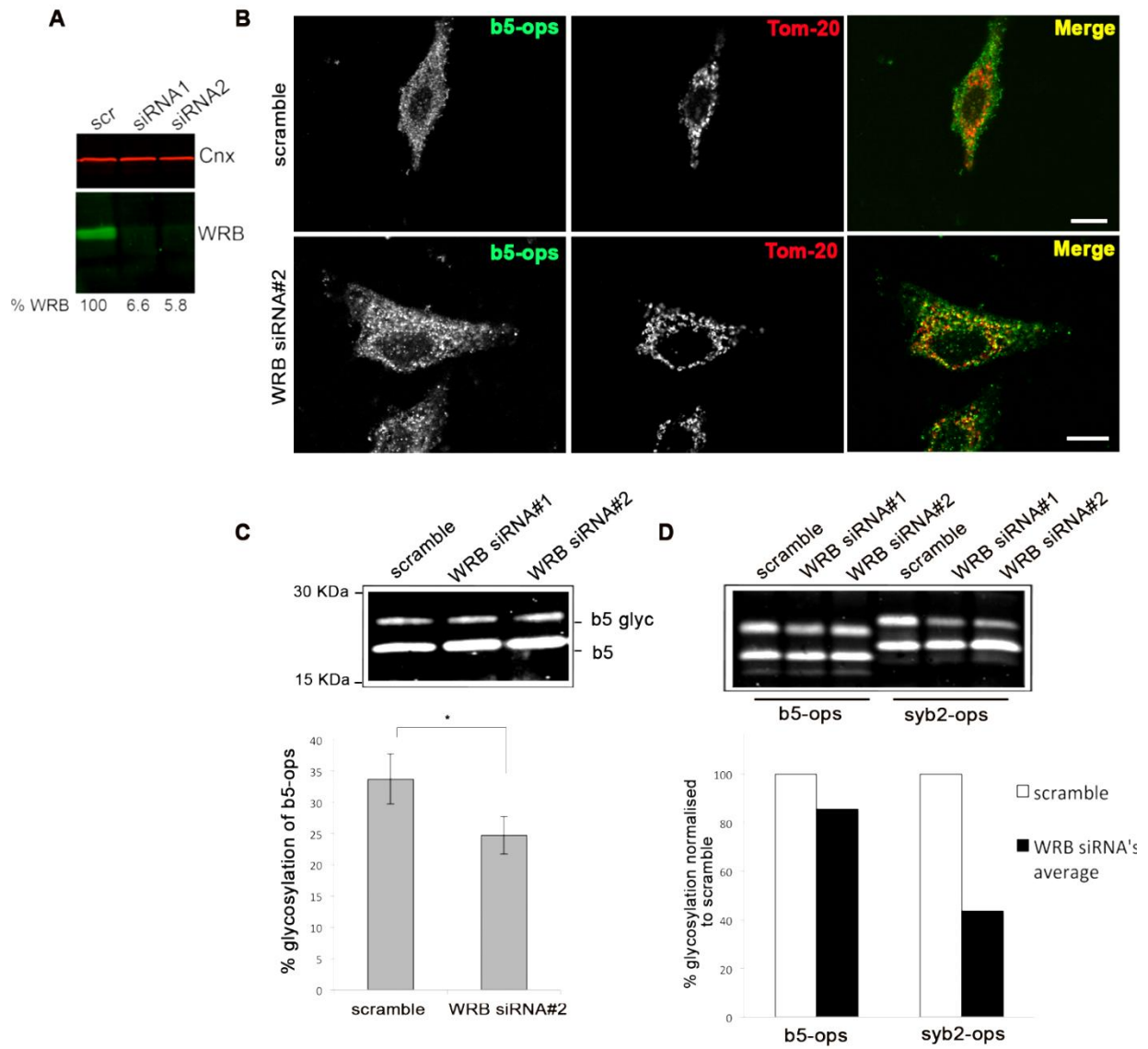
Figure 10: Effect of WRB knockdown on b5-ops insertion.

A) Lysates were prepared 48 hours after transfection with WRB silencing (siRNA1 and siRNA2) or scrambled paired oligonucleotides (scr). Equal aliquots were analysed by western blotting. Calnexin was also determined and was used for normalisation. The percentage of WRB after silencing is indicated.

B) Localisation of b5-ops in semi-intact cells after WRB knockdown. RRL and recombinant b5-ops were added to permeabilised cells transfected with scramble (upper panel) or WRB siRNA#2 (bottom panel). The cells were processed for immunofluorescence with anti-opsin antibody (green) and anti-Tom20 (MOM marker, in red). Images were acquired with the Ultraview Confocal Spinning Disk LCI (Perkin Elmer). Single sections are shown. Scale bar = 10 µm.

C) Representative western blot analysing recombinant b5-ops glycosylation in semi-intact cells silenced for WRB. Equal amounts of cell extracts were loaded, and the transferred proteins were immunoprobed with anti-opsin mAbs. The band corresponding to glycosylated b5-ER is indicated (b5 glyc). Quantification of the percentage of glycosylation (for WRB siRNA#2) is shown in the graph below. Data are presented as mean ± SEM; statistical significance was assessed by Student's paired t test (n = 4).

D) Western blot of *in vitro* translated b5-ops and syb2-ops in semi-intact cells silenced for WRB. Equal amounts of cell extracts were probed with anti-opsin mAbs. Quantification of the percentage of glycosylation (average of WRB siRNA#1 and siRNA#2) is shown in the graph below.
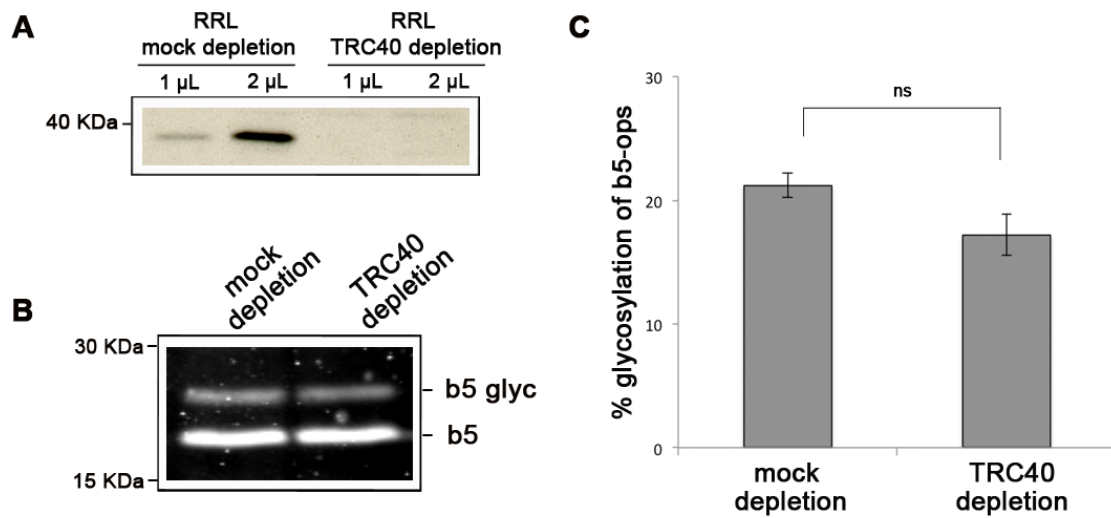
Figure 11: Effect of TRC40 depletion on b5-ops insertion.

A) Western blot analysis of 1 and 2 μl of mock- or TRC40-depleted RRL, as indicated. The blots were probed with anti-TRC40 antibodies and developed by ECL.

B) Representative western blot analysing recombinant b5-ops glycosylation in semi-intact cells incubated with ΔTRC40 RRL. Equal amounts of cell extract were immunoprobed with anti-opsin mAbs. The band corresponding to glycosylated b5-ops is indicated (b5 glyc).

C) Quantification of the percentage of b5-ops glycosylation in ΔTRC40 RRL, compared to control (mock depletion) (related to B). Data are presented as mean ± SEM (n=3).

A

scramble | SGTA siRNA#1

anti-CNX

anti-SGTA

% SGTA   100   15

B

Microinjection of b5
recombinant protein

15 min 37°C

FIX

C

b5-ER   Tom 20   Merge

scramble

SGTA siRNA#1

D

Tom20 colocalisation

ns

scramble   siSGTA#2
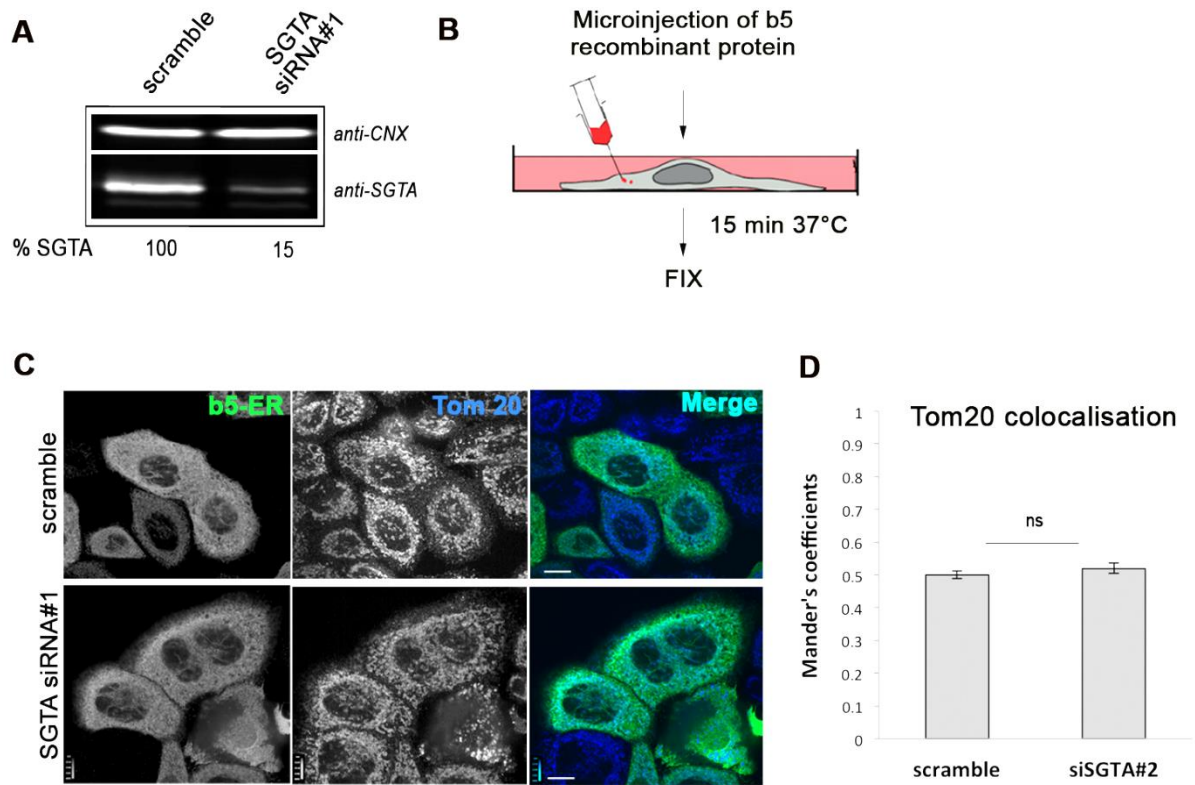
## Figure 12: Lack of effect of SGTA knockdown on b5-ER localisation.

A) Lysates were prepared 48 hours after transfection with SGTA silencing (SGTA siRNA#1) or scrambled paired oligonucleotides (scramble). Equal aliquots were analysed by western blotting and normalised to calnexin. The percentage of SGTA after silencing is indicated.

B) Scheme of the protocol used for microinjection. Cells transfected with scramble or SGTA siRNA#1 were microinjected with purified recombinant b5-ER, and then fixed after 15 minutes.

C) Localisation of microinjected b5-ER in cells silenced for SGTA. The cells were processed for immunofluorescence with anti-b5 antibody (green) and anti-Tom20 (MOM marker, in blue). Single section, acquired with the Ultraview Confocal Spinning Disk are shown. Scale bar = 10 μm.

D) Quantitative analysis of the co-localisation of microinjected b5-ER with the MOM marker, Tom20, in scramble and SGTA silenced cells. Co-localisation of b5-ER with mitochondria was quantified by Mander's co-localisation coefficients (see Methods). Data presented are mean ± SEM (scramble, n = 20 cells; SGTA siRNA#1, n = 22 cells).
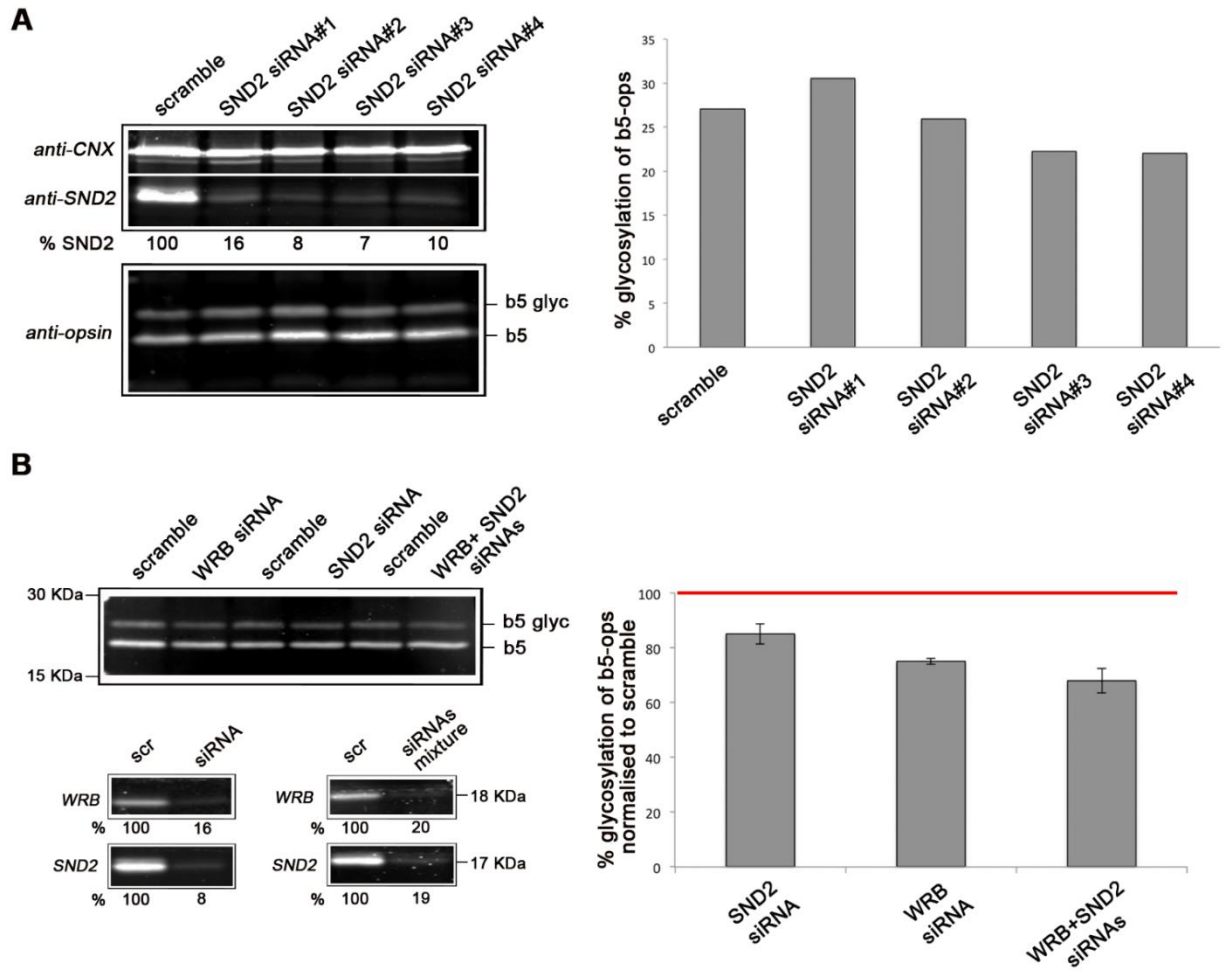
Figure 13: Effect of SND2 knockdown on insertion of b5-ops.

A) Lysates were prepared 72 hours after transfection with SND2 silencing (siRNA#1, siRNA#2, siRNA#3, siRNA#4) or scrambled paired oligonucleotides (scramble). Equal amounts of protein were analysed by WB. The percentage of SND2 after silencing (normalised to Calnexin, shown above) is indicated under the lanes. The bottom panel shows a western blot analysing recombinant b5-ops glycosylation in semi-intact cells silenced for SND2 (siRNA#1, siRNA#2, siRNA#3, siRNA#4) probed with anti-opsin mAbs. The band corresponding to glycosylated b5-ER is indicated (b5 glyc). Quantification of the percentage of glycosylation is shown in the graph on the right.

B) SND2 and WRB double knockdown. Left, upper: WB analysis of recombinant b5-ops glycosylation in semi-intact cells silenced for WRB (WRB siRNA#2), SND2 (SND2 siRNA#3), and WRB+Snd2 (WRB siRNA#2 + SND2 siRNA#3), as indicated. Left, bottom: analysis of degree of silencing of WRB or SND2, when silenced singly (left) or together (right). The graph on the right shows the quantification of the percentage of glycosylation of b5-ops under the different conditions, each one normalised to scramble siRNA. Data are presented as mean ± half-range (n=2).

A

MAL3-101   MAL2-11B

B

C

Eeyarestatin I
(ES I)

ES 35

D

E
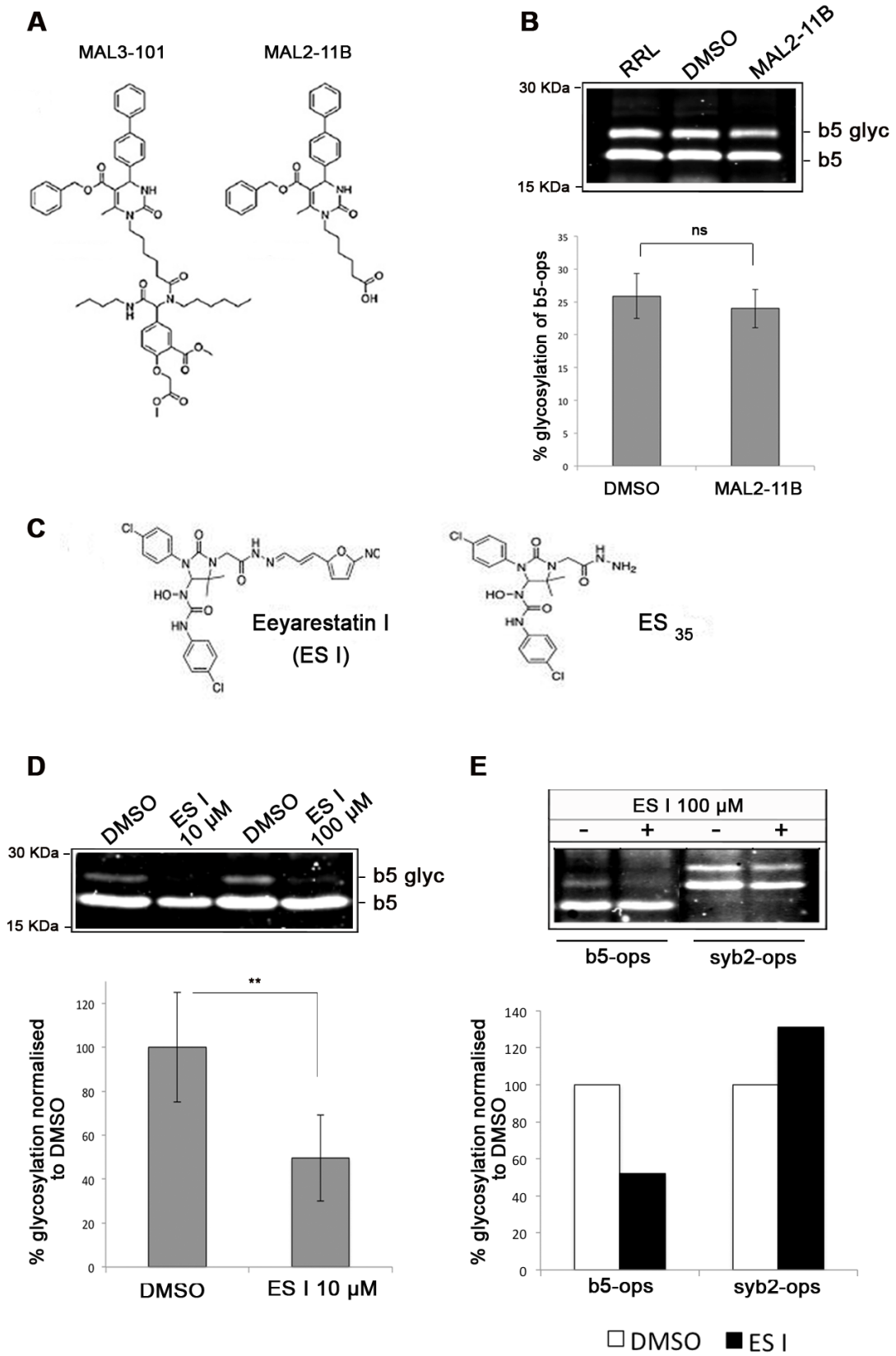
Figure 14: Treatment of RRL with drugs and its effect on ER insertion of b5-ops.

**A)** Chemical structures of the Hsp70 inhibitor MAL2-11B, derived from MAL3-101 (Wright *et al.*, 2009).

B) Representative western blot of recombinant b5-ops incubated with semi-intact cells in the presence of RRL pre-treated with 100 µM MAL2-11B. Treatment with an equal volume of DMSO was used as control. The transferred proteins were probed with anti-opsin mAbs. The band corresponding to the glycosylated b5-ops is indicated (b5 glyc). Quantification of the percentage of glycosylation of b5-ops, compared to control (DMSO) is shown in the graph on the right. Data are presented as mean ± SEM (n = 5).

C) Chemical structures of ES I and its inactive analogue, ES35 (Cross *et al.*, 2009).

D) Representative western blot of recombinant b5-ops incubated with semi-intact cells in the presence of RRL pre-treated with 10 or 100 µM ES I. Treatment with an equal volume of DMSO was used as control. The transferred proteins were probed with anti-opsin mAbs (gel on the left). The band corresponding to the glycosylated b5-ops is indicated (b5 glyc). Quantification of the percentage of glycosylation of b5-ops, normalised to control (DMSO) is shown in the graph below. Statistical significance was assessed by Student's paired t test. Data are presented as mean ± SEM (n = 4).

E) Western blot of *in vitro* translated proteins, b5-ops and syb2-ops, which were treated with 100 µM of ES I after translation and then incubated with semi-intact cells. Quantification of the percentage of glycosylation of the two proteins, normalised to control (DMSO) is shown in the graph below. Drug treatment affects b5, but not Syb2, insertion.
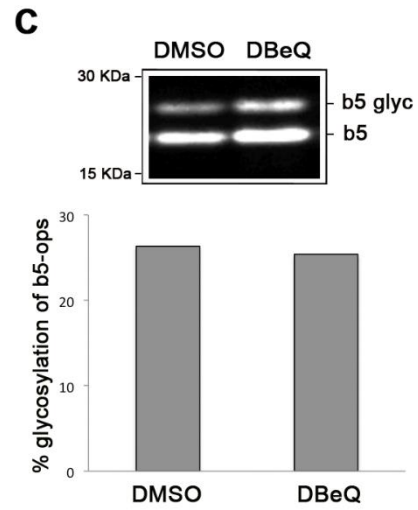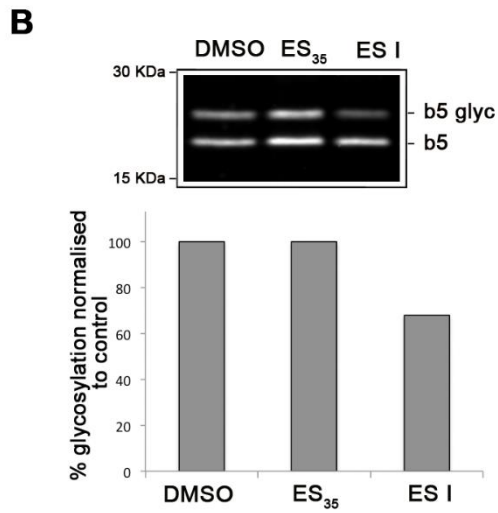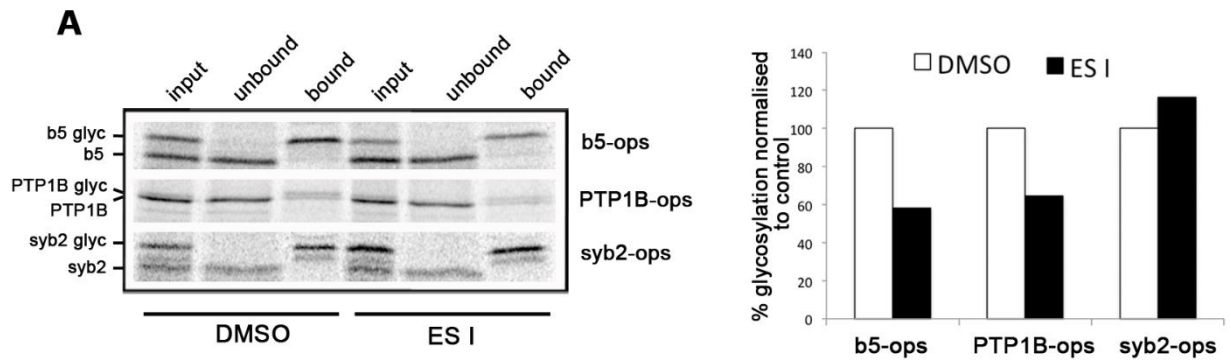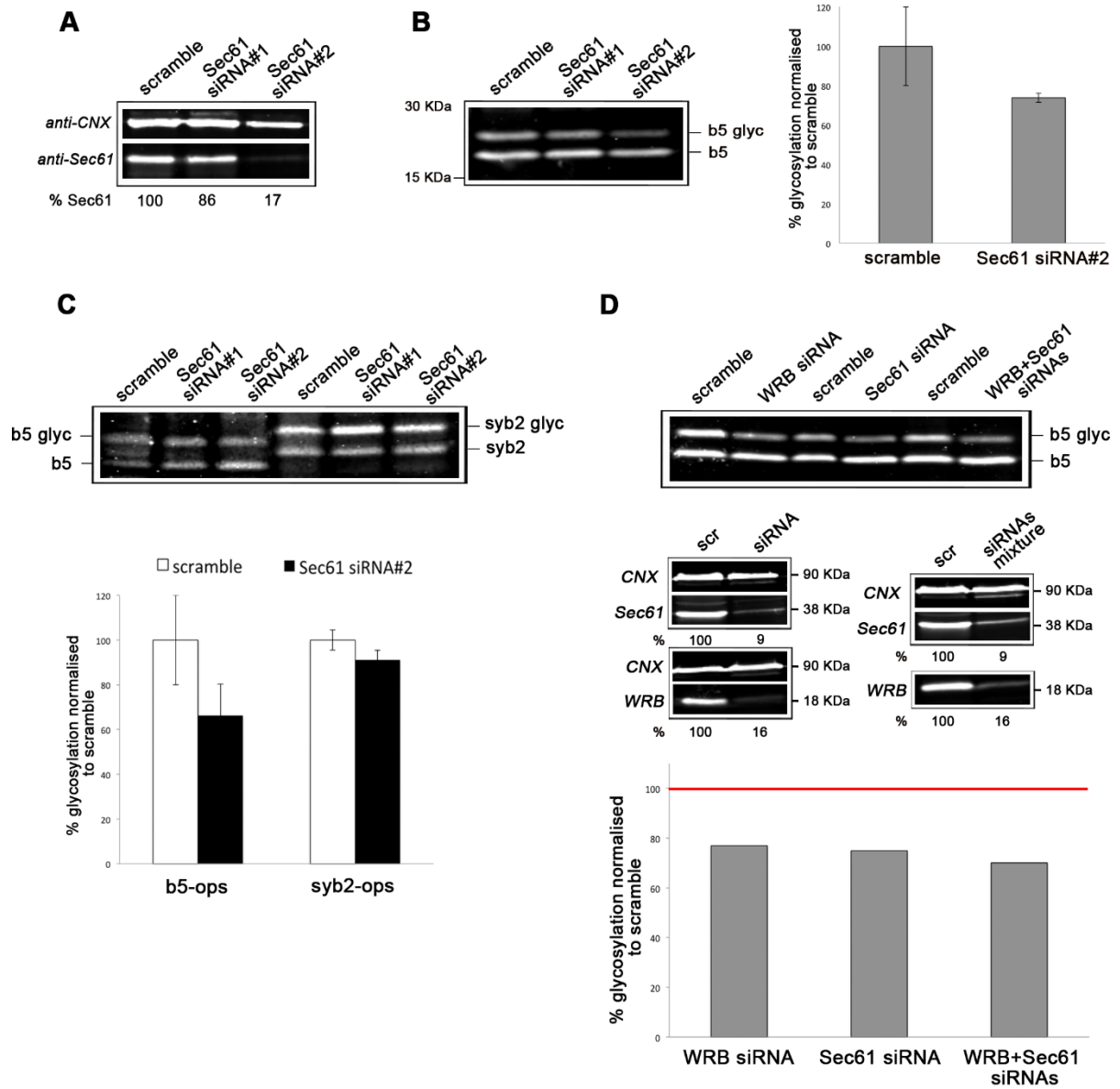
Figure 15: Effect of ES I on the ER insertion of TA proteins.

A) *In vitro* translated ^{35}S-radiolabeled, b5-ops, syb2-ops or PTP1B-ops were treated with 20 µM ES I after translation, and then incubated with semi-intact cells. Treatment with an equal volume of DMSO was used as control. Cell extracts were exposed to Concanavalin A beads, and equal aliquots of the bound and unbound fractions were loaded (see methods). 50% of the input sample was also loaded. The band corresponding to the glycosylated form of each protein is indicated (b5-glyc, PTP1B-glyc and syb2-glyc). Quantification of the percentage of glycosylation, normalised to control (DMSO) is shown in the graph on the right. Band intensities were quantified with Image Quant TL 7.0 software.

B) Western blot of recombinant b5-ops incubated with semi-intact cells in the presence of RRL pre-treated with 20 µM ES I or with its inactive analogue, ES35. Treatment with an equal volume of DMSO was used as control. The transferred proteins were probed with anti-opsin mAb. The band corresponding to glycosylated b5-ops is indicated (b5 glyc). Quantification of the percentage of glycosylation of b5-ops, normalised to control (DMSO) is shown in the graph below.

C) Western blot of recombinant b5-ops incubated with semi-intact cells in the presence of RRL pre-treated with 20 µM of DBeQ, a p97 ATPase inhibitor. Treatment with an equal volume of DMSO was used as control. The transferred proteins were probed with anti-opsin mAbs. The band corresponding to the glycosylated of b5-ops is indicated (b5 glyc). Quantification of the percentage of glycosylation of b5-ops, normalised to control (DMSO) is shown in the graph below.

Figure 16: Effect of Sec61 knockdown on the ER insertion of TA proteins.

A) Lysates were prepared 72 hours after a double transfection (at 24 and 48 hours) with Sec61 silencing (siRNA#1: SEC61A1/SEC61A2 and siRNA#2: SEC61A1) or scrambled paired oligonucleotides (scramble). Equal amounts of protein were analysed by western blotting. The percentage of Sec61 after silencing is indicated.

B) Representative western blot of recombinant b5-ops after incubation with semi-intact cells silenced for Sec61 (Sec61 siRNA#1 and Sec61 siRNA#2) in the presence of untreated RRL. The transferred proteins were probed with anti-opsin mAbs. The band corresponding to glycosylated b5-ops is indicated (b5 glyc). Quantification of the percentage of glycosylation of b5-ops, normalised to control (scramble) is shown in the graph on the right (mean ± SEM, n = 4).

C) Representative western blot of *in vitro* translated b5-ops and syb2-ops in semi-intact cells silenced for Sec61. The transferred proteins were probed with anti-opsin mAbs. Quantification of the percentage of glycosylation for Sec61 siRNA#2 knockdown, normalised to scramble, is shown in the graph below. (mean ± SEM, n = 3).

D) Sec61 and WRB double knockdown. Upper: WB of recombinant b5-ops incubated with semi-intact cells silenced for WRB (WRB siRNA#2), Sec61 (Sec61 siRNA#2), and WRB+Sec61 (WRB siRNA#2 + Sec61 siRNA#2), as indicated. The transferred proteins were probed with anti-opsin mAbs. The band corresponding to glycosylated b5-ops is indicated (b5 glyc). Middle: analysis of degree of silencing of Sec62 or WRB, when silenced singly (left) or together (right). The efficiency of silencing of both WRB and Sec61, normalised to calnexin, is shown. Bottom: Quantification of the percentage of glycosylation of b5-ops, normalised to scramble, under the indicated conditions.

7. Discussion

Although the past few years have seen a surge of interest in the biogenesis of TA proteins and a consequent impressive increase of information, especially on the TRC/GET pathway (see (Borgese N., 2015) for a recent review), many aspects of the processes by which newly synthesized TA substrates are delivered to their target membrane are still mysterious. Given the physiological importance of many members of this class of membrane proteins, for example as regulators of apoptosis or of vesicular transport and exocytosis, the complete elucidation of the pathways governing their targeting and biogenesis is of paramount importance. Notably, these biogenetic pathways could represent novel pharmacological targets, e.g., by altering the membrane insertion of members of the Bcl-2 family in cancer cells. An example of a pharmacological approach targeted to protein biogenesis/trafficking is offered by the so-called cotransins, which specifically interfere with the delivery to the ER lumen of a small number of secretory proteins (Garrison *et al.*, 2005).

One gap in our knowledge of TA protein targeting is the comprehension of pathways additional or alternative to the one mediated by TRC40. Indeed, although cell-free experiments have highlighted the importance of the TRC/GET pathway, *in vivo* or *in cellula* studies have revealed that alternative mechanisms may guarantee the delivery of many TA substrates to the ER in the absence of a functional GET pathway (Cui *et al.*, 2015; Norlin *et al.*, 2016). To investigate possible alternative pathways, I chose as model cytochrome b5, a protein that in cell-free systems spontaneously inserts into liposomes and into ER-derived microsomes in the absence of any cytosolic factor (Brambillasca *et al.*, 2005; Colombo *et al.*, 2009).

Two forms of b5 exist, which are each specifically targeted either to the ER or to the MOM (D'Arrigo *et al.*, 1993). Characterisation of the targeting signals of the two isoforms led to the conclusion that the targeting information was in the C-terminal tail (De Silvestris *et al.*, 1995); further research demonstrated that substitution of the last seven polar residues of b5-ER with two arginines (to obtain the protein b5-RR) was sufficient to reroute the protein to the MOM (Borgese *et al.*, 2001). Although b5-RR is very precisely targeted to the MOM *in vivo*, in a cell-free system it efficiently inserts into the ER. Likewise, b5-ER, as mentioned above, can insert into protein-free liposomes, provided that they have low

cholesterol content. Each of the two proteins, when microinjected into the cytoplasm of cultured cells, inserts into its cognate organelle, indicating that the protein itself contains the information for correct post-translational targeting (unpublished). The mysterious pathways followed *in vivo* to achieve precise subcellular localisation of the two b5 forms have been the subject of my thesis research.

Given the distinct differences in the *in cellula* and cell-free behaviour of the two b5 forms, it was clear that not much information in addition to what is already known could be obtained from classical cell-free systems. I therefore decided to utilise a system that can be considered intermediate between cell-free and *in cellula*, consisting of semi-permeabilised cells; the plasma membrane of these cells are made permeable to macromolecules by treatment with low concentrations of digitonin, but the intracellular membranes remain intact (Wilson and Bulleid, 2000; Setoguchi *et al.*, 2006). With this system, it is possible to replace the endogenous cytosol with cytosol from an exogenous source manipulated as desired, e.g., by removing or adding components. Compared to cell-free systems, these cells present the advantage of having all their endogenous organelles in the correct spatial relationships. In this system, it is also possible to carry out both biochemical and morphological assays. In my research, I combined the use of semi-permeabilised cells with silencing experiments, and also with microinjection. Using these approaches, I was able to obtain novel and unexpected information on the targeting pathways followed by the two b5 forms, which I discuss in this section of my thesis.

The MOM as default destination of TA proteins.

Previous work from my laboratory has demonstrated that both forms of b5 can insert into protein-free liposomes, provided that they have a very low cholesterol content; the inclusion of cholesterol in a ratio to phospholipids as low as 0.1 (Brambillasca *et al.*, 2005) nearly completely blocks b5 insertion. The negative effect of the sterol could explain why TA proteins don't insert into membranes of the secretory pathway downstream to the ER, as these have high cholesterol content well above the 0.1 threshold value (Holthuis and Menon, 2014). This explanation leaves open, however, the mechanism by which TA proteins

discriminate between low cholesterol containing membranes, i.e., in metazoans and fungi the ER, the MOM and peroxisomes.

Based on the data from my laboratory showing that both b5-ER and b5-RR can insert efficiently both into ER-derived microsomes and into protein-free liposomes, we expected that in semi-permeabilised cells, and in the absence of cytosol, both forms would be similarly promiscuous. We were therefore surprised to observe that, in buffer alone, both b5 forms quite specifically targeted the MOM and were apparently completely excluded from the ER. The addition of cytosol and a source of energy, while not affecting the mitochondrial localisation of b5-RR, rerouted b5-ER to its correct destination. These results indicate that, although in cell-free systems both b5 forms can insert into ER membranes, when faced with a choice, and in the absence of cytosolic factors, the MOM is the preferred destination for both of them. It may be that translocation of the C-terminus of TA proteins across the MOM is more rapid than across the ER; one can imagine that initially the TA substrate samples different bilayers, but that once its C-terminus is translocated, its localisation is irreversibly determined. In the absence of cytosol, the MOM might win the competition on a kinetic basis, in that translocation across its bilayer could be more rapid than across the bilayer of other organelles. Instead, in the presence of cytosol, targeting factors that do not recognise the b5-RR form, would capture b5-ER, preventing it from inserting into the MOM and allowing its insertion into the ER instead. It must be mentioned that the insertion of TA proteins in buffer alone is possible only for those with moderately hydrophobic TMDs; those with more hydrophobic TMDs (like synaptobrevin 2) are insoluble in the absence of chaperones, and therefore not amenable to targeting studies under buffer-only conditions.

Previous studies have led to the idea that specific chaperones are required to route TA proteins to the ER and to peroxisomes, and that the MOM represents the default destination of TA proteins, i.e., the membrane into which they insert in the absence of interaction with specific targeting chaperones. This idea is based on a number of observations: (i) in yeast, a number of GET substrates are partially localised to mitochondria in ΔGET strains (Schuldiner et al., 2008); (ii) many MOM TA proteins have moderately hydrophobic TMDs, and can spontaneously insert into liposomes (Kemper et al., 2008); this feature keeps them from

interacting with Sgt2 and from the subsequent delivery to Get3. Indeed, increasing the hydrophobicity of a MOM TA protein (Fis1) resulted in its interaction with components of the GET pathway (Wang *et al.*, 2010); (iii) no cytosolic nor MOM protein have so far been identified as components of a pathway for MOM delivery (Setoguchi *et al.*, 2006; Krumpe *et al.*, 2012); iv) similarly, the Pex19-dependent targeting of peroxisomal TA proteins depends on an excess of positively charged residues in the tail region; the feature of a positively charged tail region is shared by MOM proteins, but the amount of positive charge is more marked in peroxisomal substrates and required for delivery to the peroxisome; reduction of this charge results in mislocalisation to the MOM (Yagita *et al.*, 2013).

Although the work described in the previous paragraph, taken together, did suggest that the MOM could be a default destination, a clear-cut result like the one presented in this thesis had not yet been obtained. My results give strong support to the hypothesis of the MOM as default destination, in that I show that in the absence of cytosol, an ER TA protein is dramatically rerouted to mitochondria. This result raises two questions: (i) what is the feature of the MOM that favours it as destination of TA proteins over other intracellular membranes; and (ii) what is the identity of the molecular chaperones required for the ER targeting of b5-ER?

Concerning the first question, previous studies have excluded the participation of the TOM machinery ((Setoguchi *et al.*, 2006; Kemper *et al.*, 2008, Krumpe *et al.*, 2012) see introduction). Furthermore, treatment of semi-permeabilised cells with proteolytic enzymes, resulting in the digestion of proteins exposed on the MOM surface, did not affect the targeting of investigated TA substrates (Bak, Bcl-XL, and Omp25). On the other hand, it has been suggested that the low sterol content of the MOM may underlie its capacity to integrate TA proteins (Krumpe *et al.*, 2012). While this hypothesis may be valid for yeast cells, in which the MOM does appear to have significantly lower sterol content than the ER membrane (Zinser *et al.*, 1991; Schneiter *et al.*, 1999), in mammals, the limited number of studies have indicated that the sterol content of the MOM and the ER are quite similar (Colbeau *et al.*, 1971), or actually higher in the MOM than in the ER (Wibo *et al.*, 1981). Thus, at least in mammals, the permissivity of the MOM must depend on a factor different from

sterol content: either on an embedded protein not accessible to proteases on the cytosolic side of the membrane, or on differences in composition of lipids other than sterols. As a possible candidate, I consider cardiolipin, a lipid exclusive to mitochondria, present mainly on the inner mitochondrial membrane but also in lower amounts on the MOM, whereas it is particularly enriched at contact sites between the inner and outer membranes (Hovius *et al.*, 1990; Lutter *et al.*, 2000). So far, however, my attempts to reveal alterations in MOM TA protein localisation in cardiolipin synthase silenced cells have been unsuccessful (result not shown in this thesis).

The second question (identity of molecular chaperones required for the ER targeting of b5-ER) is addressed in the following section of this Discussion.

Molecular chaperones involved in the ER insertion of b5-ER.

In cell-free systems it has been amply demonstrated that b5 insertion into ER-derived microsomes is independent from the TRC40 pathway: immunodepletion of TRC40 from the RRL, while strongly inhibiting insertion of some substrates (e.g., Synaptobrevin-2 (Colombo *et al.*, 2009); Sec61β (Rabu *et al.*, 2009)), is completely ineffective on b5 insertion (Colombo *et al.*, 2009); furthermore, inclusion in post-translational insertion assays of WRBcc, which inhibits TRC40-mediated targeting by acting as decoy receptor (Vilardi *et al.*, 2011), is ineffective on b5 insertion (Colombo *et al.*, 2016). Nevertheless, since b5's behaviour in cell-free assays does not reflect its targeting behaviour *in cellula*, I considered it likely that the TRC40 pathway could play an important role in its discrimination between the ER and the MOM *in vivo*, and in semi-intact cells in the presence of cytosol. This supposition was supported both by demonstration of the energy dependence of the targeting phenomenon (Figure 3), and by the previous observation that b5-ER does weakly interact with TRC40, as assessed by pulldown experiments (Colombo *et al.*, 2009). I therefore put a lot of effort into analysing the effect of interference with the TRC40 pathway on b5 integration into the ER in my system.

I investigated a possible involvement of the TRC40 pathway in semi-permeabilised cells by three different approaches: (i) inclusion of WRBcc in the assay; (ii) the use of WRB-

silenced cells in the assay; (iii) immunodepletion of TRC40 from the RRL. Since small differences in the intracellular distribution of b5-ER would have been difficult to assess quantitatively by IF, in all three of these approaches, I used glycosylation of b5-ops as reporter for ER insertion. The results showed, that differently from the previous observations in cell-free systems, interference with the TRC40 pathway did diminish ER targeting of our substrate, however, this effect was partial (20-30%), and more marked for the recombinant protein than for the *in vitro* translated product. As positive control, I also analysed the effect of the treatments on the insertion of Syb2-ops, and found, as expected, near complete inhibition by WRBcc and 60% inhibition by WRB silencing. I also investigated the possible effect of the first member of the TRC40 pathway, SGTA (see Introduction), by microinjecting recombinant b5 into cells with downregulated SGTA and then analysing the microinjected cells by IF; by this approach I did not detect any difference between SGTA-silenced and control cells. The role of SGTA remains, however, to be further investigated, since, as mentioned above, small differences in subcellular localisation of b5 might not be detected by IF. From my results, I can conclude that the TRC40 pathway represents a delivery route to the ER for b5; however, the results also indicate that additional pathways must be available to this substrate, as the majority of the protein in any case was able to reach its correct destination in the absence of a functional TRC40 pathway.

In search of these additional pathways, I investigated a possible involvement of Hsc70, the inhibition of which, in cell-free systems, was found to interfere with b5 insertion (Rabu *et al.*, 2008). To this end, we applied a number of small molecule inhibitors of Hsc70, pyrimidone-peptoid compounds derived from MAL3-101, with different mechanisms of action (Wright *et al.*, 2008). MAL2-11B and DMT003024 are both potent inhibitors of the Hsp40-stimulated Hsp70 ATPase activity, while DMT002220 interferes with both endogenous and Hsp40-stimulated Hsp70 ATPase activities. However, we found no effect of any of these inhibitors. The observed effects in the cell-free system might be indirect, due to aggregation of other Hsc70 clients, which could lead to non-specific co-aggregation of the *in vitro* synthesized b5 product. Thus, while Hsc70 may aid solubilisation of newly synthesised b5, it appears that the protein can faithfully target the ER in the absence of the

function of this chaperone. Likewise, inhibition of another important cytosolic chaperone, Hsp90, was also without effect on b5-ops glycosylation.

Another pathway that I probed is the recently described SND pathway (Ast *et al.*, 2013; Aviram *et al.*, 2016). This pathway, characterised in yeast, is reported to consist of a cytosolic protein and two ER integral membrane proteins, and is mainly involved in targeting membrane proteins with internal TMDs that escape SRP. Its function overlaps with the SRP pathway, on the one hand, and with the GET pathway, on the other. In mammals, only a homologue of one of the ER proteins is present (SND2), and antibodies to this protein were provided by Richard Zimmermann (University of Saarland, Germany). I silenced SND2 with four different oligonucleotide pairs, but obtained at most a non-statistically significant ~10% inhibition on b5-ER insertion in the silenced cells. Reasoning that the lack of effect of SND2 silencing could be due to compensation by the TRC40 pathway, I investigated the effect of silencing both pathways together; however, I found that SND2 silencing had very little effect over WRB silencing alone. Thus, I conclude that the SND pathway is not involved in b5 targeting.

Among the small molecule inhibitors that I tested in my assay, one, ES I, had a marked effect on b5-ER insertion, causing, at concentrations routinely used (10 - 100 µM), a reduction of up to 60% in the glycosylation of both the recombinant and the *in vitro* translated product. Quite strikingly, it was without effect on the TRC40-dependent substrate, Syb2, demonstrating that the process of glycosylation itself is not affected by ES I, and was equally effective on another TA substrate capable of unassisted insertion into protein-free liposomes (PTP1B, see Introduction). Thus, ES I seems to specifically target an ER targeting pathway used by spontaneously inserting TA proteins.

As explained in the Introduction, ES I interferes with a number of processes occurring at the ER. It is best known as an ERAD inhibitor, which it inhibits by associating with p97. p97 (also known as valosin-containing protein (VCP) is an AAA-ATPase (ATPase Associated with various cellular Activities) that plays a central role in the extraction of substrates from the ER lumen to the cytosol and their subsequent delivery to the proteasome. The association of ES I with p97 inhibits the activity of a p97-dependent deubiquitinating enzyme required

for completion of ERAD (Wang *et al.*, 2008). To test whether p97 is involved in the response of b5 targeting, I applied a specific inhibitor of p97's ATPase activity, DeBQ. The observation that this compound was without effect makes it unlikely that p97 inhibition underlies the effect of ES I on b5 and PTP1B insertion.

In addition to its effect on ERAD, ES I also inhibits the Sec61 translocon (Cross *et al.*, 2009). Although it is known that Sec61 plays no role in the insertion of any TA protein into ER-derived microsomes in cell-free systems, we tested a possible involvement of Sec61 in our system, again comparing the effect of Sec61 downregulation on b5-ops and Syb2-ops glycosylation. Remarkably, I found that Sec61 silencing had an effect comparable to the one of WRB depletion on b5-ops, but not on Syb2-ops, glycosylation, indicating an unexpected role of the translocon in insertion of a spontaneously inserting TA protein, while again excluding its role in the insertion of a classical TRC40-dependent substrate (Syb2). The result further suggests that of ES I inhibition may be due to the effect of the inhibitor on the translocon. Nevertheless, it should also be kept in mind that, due to the stability of the translocon, the silencing of Sec61 takes a full four days, and that I obtained efficient silencing with only one oligonucleotide pair. At present, I cannot therefore exclude off-target effects of this siRNA or, more likely, effects secondary to Sec61 depletion. To investigate the relationship between the inhibitory effects of ES I and Sec61 downregulation, I am currently in the process of testing the effect of ES I on Sec61-silenced cells.

Since my results uncovered two ER proteins, WRB and Sec61, as important players in b5-ER targeting/insertion, I asked what would happen if both these proteins were downregulated together. My reasoning was that, if each of them were operating in a distinct pathway, and if these two pathways represented the only available ones for b5-ER targeting, then concurrent blockage of both would result in increased reduction of b5-ER targeting, or even in potentiation of the inhibition. Instead, I found that cells downregulated for WRB and Sec61 were no less proficient in b5-ops glycosylation than the single silenced cells. There are two possible interpretations of this result: either TRC40/WRB and Sec61 are acting in the same pathway, so that regardless of whether each or both of them are silenced, the entire pathway is blocked; or the two pathways are different, but compensatory targeting

mechanisms come into play when both are downregulated. In either case, it is clear that pathways additional to the TRC40 and/or Sec61 pathway operate, as complete or near to complete inhibition of b5-ER targeting was never achieved in my system.

Conclusions and Future Directions

In the described research, I have used cyt b5 as model TA protein to investigate poorly understood post-translational targeting mechanisms. Although my work has not uncovered all the pathways that guarantee robust targeting of this protein, it has generated important contributions to a problem important for cell biology, physiology, and pathology, as well.

First, my work shows directly that the default destination for a spontaneously inserting ER-targeted TA protein is represented by the MOM. This result is in agreement with previous speculations based on indirect evidence. What features of the MOM are responsible for this is at present unclear. There are various approaches that could be undertaken to investigate this phenomenon, all rather risky and labor-intensive. One might start with a purified MOM fraction (which is difficult to obtain in large quantities and of sufficient purity), and compare the kinetics of b5-ER insertion into the membranes of this fraction with the rather slow kinetics for insertion into ER microsomes. If, as hypothesized, the rate for insertion into the MOM is more rapid than into the ER, this faster insertion could be used as readout to evaluate the contribution of proteins and/or lipids of the MOM in the insertion process.

Second, my work reveals that targeting of spontaneously inserting proteins to the ER is energy and chaperone-dependent, and that multiple pathways co-exist, which guarantee robust targeting of the substrates. Future research may identify the pathways that my research suggests do exist but that I have not yet identified. Again, this is a difficult task because of the redundancy of the pathways. Thus, an unbiased screening approach might fail to give any meaningful hit. One possible (labor-intensive) approach could be to use semi-permeabilised, doubly silenced WRB/Sec61 cells in assays with RRL fractionated by various techniques, in an attempt to identify the cytosolic fraction that is active in the absence of the two ER receptors.

Interestingly, the additional pathways responsible for b5 targeting do not appear to be available to TA proteins with more hydrophobic TMDs, at least not in the system of semi-intact cells that I used. Indeed, interference with the TRC40 pathway essentially shut off Syb2-ops insertion, implying that it did not have access to the pathways that b5-ER was using. Conversely, treatments that affected b5-ER insertion (ES I treatment and Sec61 downregulation) had no effect on the targeting of Syb2. Thus, different ER-targeted TA proteins rely on different targeting pathways. This situation contrasts with that of co-translational protein insertion, which mainly relies on the SRP pathway. In conclusion, my results reveal redundant, but distinct pathways in post-translational targeting of different TA proteins to the ER, and open the way to the complete unraveling of the complexities of this process.

8. List of abbreviations

AAA-ATPase	ATPase associated with various cellular activities
ATP	Adenosine triphosphate
b5	Cytochrome b_5
B5-ER	ER-targeted form of b5
B5-RR	MOM-targeted form of b5
BAM	β-barrel assembly machinery
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CAML	Calcium modulating cyclophilin ligand
CBB	Coomassie brilliant blue
CMV	Cytomegalovirus
COE	Chloroplast outer envelope
ConA	Concanavalin A
CPY	Carboxypeptidase Y
CTS	Chloroplast targeting sequence
DB	Denaturation buffer
DMEM	Dulbecco's modified Eagle's medium
EM	Electron microscopy
ER	Endoplasmic reticulum
ERAD	ER associated degradation
ES I	Eeyarestatin I
FBS	Fetal bovine serum
GDB	Gelatin dilution buffer
GET	Guided entry of tail-anchored proteins

GS4B	Glutathione-sepharose 4B resin
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
HS	High salt buffer
IF	Immunofluorescence
IMP	Integral membrane protein
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
LB	Luria Bertani
mAb	Monoclonal antibody
MBP	Maltose binding protein
MFF	Mitochondrial fission factor
MIM	Mitochondrial import machinery
MOM	Mitochondrial outer membrane
mPTS	Peroxisome targeting signal
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PDI	Protein disufide isomerase
PMP	Peroxisomal membrane protein
PTP1B	Protein tyrosine phosphatase 1B
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNC	Ribosome-nascent chain complex
ROI	Region of interest
RRL	Rabbit reticulocyte lysate
RT	Room temperature
SAM	Sorting and assembly machinery
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean

siRNA	Small interference RNA
SNARE	Soluble NSF attachment receptor
SND	SRP-independent
SR	SRP receptor
SRP	Signal recognition particle
SS	Signal sequence
Syb	Synaptobrevin
TA	Tail-anchored
TIM	Translocase of the inner membrane
TMD	Transmembrane domain
TOM	Translocase of the outer membrane
TPR	Tetratricopeptide repeat
TRAM	Translocating-chain associating membrane
TRAP	Translocon associated protein
TRC	Transmembrane recognition complex
UBL	Ubiquitin-like domain
VCP	Valosin-containing protein
WB	Western blot

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