Investigating the function of Ysy6p and the EMC complex

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LIST OF ABBREVIATIONS

ADP Adenosine di Phosphate

ATP Adenosine tri phosphate

CFTR Cystic Fibrosis Transmembrane Conductance Regulator

CHAPS 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

dH_2O distilled H_2O

DMSO Dimethyl Sulfoxide

DSS disuccinimidyl suberate

DTT Dithiothreitol

E. coli Escherichia coli

ER Endoplasmic Reticulum

ERAD ER associated degradation

ERES ER exit sites

FITC Fluorescein isothiocyanate

GFP Green Fluorescent Protein

GTP Guanosine tri phosphate

GDP Guanosine di phosphate

HS High salt

IMS Intermembrane Space

LS Low salt

MBS Maleimidobenzoyl-N-hydroxysuccinimide ester

NAC Nascent chain associated complex

OST Oligosaccharide Transferase Complex

PAGE polyacrylamide gel electrophoresis

PBS Phosphate buffered saline

RAMP Ribosome associated membrane protein

SAM Sorting and assembly machinery

SDS Sodium dodecyl sulphate

S. Cerevisiae Saccharomyces Cerevisiae

SPC Signal peptidase complex

SR SRP receptor

SRP Signal recognition particle

TAT Twin arginine targeting

TIC Transport at the inner membrane of the chloroplast

TOC Transport at the outer membrane of the chloroplast

TM Transmembrane Domain

TRAM Translocating chain-associating membrane protein

TRAP Translocon associated protein

UPR Unfolded protein response

UPRE Unfolded protein response element

v/v volume/volume

w/v weight/volume

ABSTRACT

During or following mRNA translation by cytosolic ribosomes the nascent protein being synthesised can be targeted to the endoplasmic reticulum (ER). This targeting of nascent proteins leads to either their transport across the ER or in the case of transmembrane proteins their integration into the ER membrane. This process is carried out by the Sec61 complex which is conserved across organisms including bacteria, yeast and mammals. The ER is a site of protein folding, modification, forward transport and quality control. Misfolded proteins can be directed to the ER associated degradation pathway which mediates retrotranslocation of proteins out of the ER and degradation by the proteasome.

A lot is already known about ER associated processes, however, the function of many factors associated with the ER are still poorly understood. Two examples of such factors in yeast include Ysy6p and the ER membrane complex (EMC). The mammalian homolog of Ysy6p, RAMP4 has been shown to be recruited to ribosomes during the integration of transmembrane proteins into the ER and implicated in processes such as protein degradation. The function of the EMC is unknown but has been suggested to function in either protein folding, forward trafficking from the ER or ERAD.

Here it has been shown that deletion of *EMC1*, *EMC2*, *EMC3*, *EMC6* but not *EMC4* or *EMC5* causes defects in growth at 39.5°C or on media containing SDS. Defective growth on media containing SDS is suggestive of defects in cell wall or membrane biogenesis and both SDS and elevated temperature are known to cause the induction of the unfolded protein response. This suggests that certain members of the EMC complex are involved in stress responses or secretory processes. Functional investigation of ER associated pathways showed that a Δ*emc5* strain has a defect in clearance of the ERAD-M substrate Hmg2-6myc however Δ*emc1* does not. The ERAD and competitive growth phenotypes are therefore unlinked and suggests that the EMC complex has a function in multiple pathways which are independently affected by deletion of individual members of the complex.

To test whether Ysy6p was a functional yeast homolog of RAMP4, processes previously observed to be involved with the function of RAMP4 were investigated. Strikingly, it has been demonstrated that Ysy6p, similarly to RAMP4, is a protein which tightly associates with ribosomes. In addition, RAMP4 has been previously observed to affect protein degradation whereas here it has been shown that deletion of *YSY6* causes an alteration in the degradation profile of Hmg2-6myc. The findings are therefore consistent with the notion that Ysy6p and RAMP4 are functional homologs.

In order to further characterise the molecular basis for ribosome association N terminal truncations of opsin tagged Ysy6p were made. It was demonstrated that partial deletion of a conserved cytosolic domain predicted to form a helix-turn-helix resulted in a near loss of cosedimentation of Ysy6p with ribosomes. Furthermore, it was shown that Ysy6p crosslinks with MBS to three factors of 22 kDa, 18 kDa and 10 kDa the identities of which remain undetermined.

The data therefore suggest that Ysy6p is involved in processes associated with co-translational integration of proteins and ERAD. In addition, the data also suggest that the EMC complex is involved in ERAD and stress responses.

DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

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DEDICATION

This thesis is dedicated to my grandfather Prof. Forbes W. Robertson who sadly passed away this year.

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Lastly, I would like to thank my family who have been missed during my time in Manchester.

CHAPTER 1 - Introduction

1.1 The secretory pathway: directing protein localisation.

Eukaryotic cells are composed of a network of functionally distinct membrane bound compartments (Lodish et al., 2000). The vast majority of protein synthesis is carried out by cytosolic ribosomes. However, in many cases these proteins need to be targeted to specific compartments for them to carry out their biological function. This is carried out by the secretory pathway.

The secretory pathway starts at protein translation where the nascent chain can stall translation and cause the ribosome to bind to the endoplasmic reticulum (ER) (Rapoport, 2007, Lodish et al., 2000). The protein can therefore be targeted to the ER in a co-translational manner. Alternatively, the protein may be synthesised in the cytosol and post-translationally transported to the ER via binding to chaperones. When targeted to the ER the nascent protein is either integrated into the ER membrane or translocated into the ER lumen. This translocating process is carried out by a translocon known as the Sec61 complex. The ER lumen constitutes an environment where proteins can undergo many modifications such as glycosylation or disulphide bond formation (Braakman and Bulleid, 2011, Zimmermann et al., 2011). In addition, the ER contains multiple chaperones which assist the correct folding of proteins. Following correct protein folding in the ER, proteins can be transported to other compartments such as the Golgi and the plasma membrane via directed vesicular transport.

The ER also targets misfolded proteins for degradation in a process known as ER Associated Degradation (ERAD) (Benyair et al., 2011). The ER therefore plays a central role in the secretory pathway by sorting, modifying and assembling proteins.

1.2 Protein targeting and integration at the Endoplasmic Reticulum

1.2.1 The diversity of protein substrates destined for the Endoplasmic Reticulum

Proteins fold into distinct structures which enable them to carry out their function. Proteins, including those targeted to the ER, are therefore topologically diverse (Skach, 2007, Ott and Lingappa, 2002). Integral membrane proteins need to be correctly

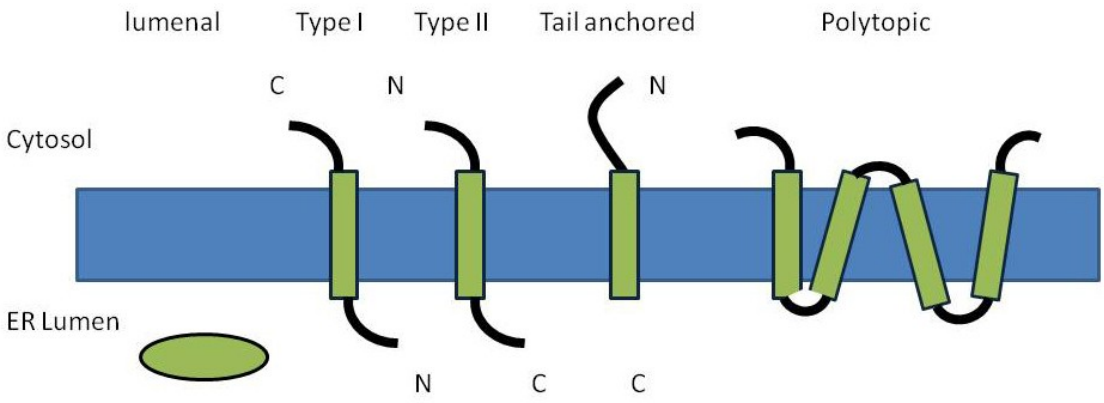


Figure 1.1 Classes of ER targeted proteins. Proteins targeted to the ER have diverse final topologies. Soluble lumenal proteins are translocated across the ER membrane. Transmembrane proteins are integrated into the ER membrane and have been classified on the basis of the number of transmembrane domains and the localisation of their N and C termini.

integrated into the ER whereas ER lumenal proteins have to be transported across the ER membrane. ER membrane proteins have been classified on the basis of the relative localisation of their N and C termini relative to the ER membrane (Figure 1.1). Type I membrane proteins contain a lumenal N terminus and cytosolic C terminus. The converse classifies type II membrane proteins. Tail anchored (TA) proteins contain a short lumenal C terminus and cytosolic N terminus and polytopic proteins span the membrane multiple times with their C or N terminus located either in the cytoplasm or ER lumen. The challenges facing the early secretory pathway are therefore to correctly integrate transmembrane proteins into the ER membrane in a manner that avoids the aggregation of their hydrophobic transmembrane segments. Correct protein topology is attained with the help of a translocase known as the Sec61p complex which can function in either a co-translational or post-translational manner (Plath et al., 2004, Görlich and Rapoport, 1993). Strikingly however, the Sec61p complex has also been shown to be sufficient for protein integration (Görlich and Rapoport, 1993). However, the existence of a secondary pathway termed the GET pathway which specifically aids the insertion of TA proteins at the ER has recently been characterised (Schuldiner et al., 2008).

1.2.2 Targeting to the ER via the co-translational pathway

In the co-translational pathway polypeptide elongation is arrested shortly after the translation of a signal sequence (Walter et al., 1981). Signal sequences are poorly conserved in sequence across proteins but their comparison enabled von Heijne (1985) to construct a minimal signal sequence. This minimal signal sequence is composed of, as shown in Figure 1.2: an N-terminal Met residue; a strictly hydrophobic segment of seven amino acids, which may at most contain one Ser, Gly, Thr or Pro residue; and a third more polar segment of five amino acids (von Heijne, 1985). The arrest in translation is caused by the binding of a protein to the ribosome known as the Signal Recognition Protein (SRP) (Walter et al., 1981). SRP is conserved across all domains of life and is composed of an RNA molecule to which proteins are recruited to form an RNA-Protein complex (Pool, 2005). This complex preassembles in a stepwise manner before mediating translational arrest (Hainzl et al., 2002, Oubridge et al., 2002, Siegel and Walter, 1988). The manner in which translational arrest occurs is still not

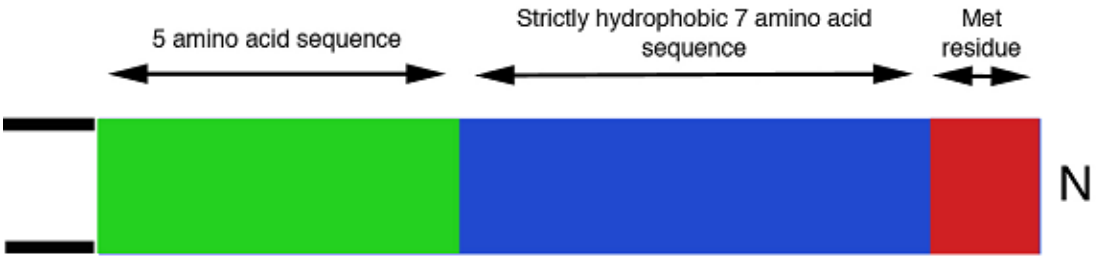


Figure 1.2 The minimal signal sequence. The minimal signal sequence constructed by von Hejine (1985) contains an N terminal met residue, a strictly hydrophobic 7 amino acid sequence (blue); and a sequence of 5 amino acids (green) which is more polar than the 7 amino acid sequence.

understood. However, it is thought that SRP binds to the ribosome close to the nascent chain exit tunnel, independently of the presence of a signal sequence (Halic et al., 2004, Flanagan et al., 2003, Pool et al., 2002, Walter et al., 1981). The emergence of the nascent chain causes SRP to bind via hydrophobic interactions to the signal sequence (Hainzl et al., 2011). In addition, the binding of SRP to the signal sequence seems to promote rearrangements in ribosome-SRP binding in a manner that has been suggested to hinder association of elongation factor 2 (EF-2) with the ribosome which rationalises translational stalling (Halic et al., 2004).

During this arrest in elongation, the complex formed by the ribosome, the nascent-chain and SRP is targeted to the ER translocon (figure 1.3). This process is mediated by three main components: the SRP protein SRP54, the translocon and the SRP receptor (SR). The SR is composed of an integral ER membrane protein, SRβ, and a peripheral membrane protein, SRα (Miller et al., 1995). SRP54, SRα and SRβ are all GTPase proteins which can bind GTP (Freymann et al., 1997, Montoya et al., 1997, Miller et al., 1995). SRP54 binding to the ribosome causes an increase in affinity of SRP54 for GTP (Bacher et al., 1996). SRα shares homology with SRP54. Both can bind GTP and SRP54 and SRα associate only when GTP is bound to both (Rapiejko and Gilmore, 1997). The crystal structure of bacterial SRP54 and SRα homologs suggest that the interaction leads to dimerisation of SRP54 and SRα via their nucleotide binding domains (Focia et al., 2004). SRβ is stably associated with SRα (Tajima et al., 1986). It is proposed that SRβ is maintained in a GDP bound state by interaction with ribosomal proteins until the Sec61p complex has been recruited to the ribosome-SRP complex. SRβ then binds GTP in a manner which promotes the release of the signal peptide from SRP (Fulga et al., 2001). Recruitment of the translocon therefore stimulates signal sequence release from SRP and its transfer to the translocon which results in GTP hydrolysis (Halic et al., 2004, Pool et al., 2002, Song et al., 2000, Bacher et al., 1996).

Contacts between the ribosome and the translocon have been mapped to the ribosomal proteins L23a and L35 (L25 and L35 in yeast) (Becker et al., 2009, Morgan et al., 2002, Beckmann et al., 2001, Menetret et al., 2000). Upon translocon binding the SRP54 protein appears to be displaced away from the L23a protein (Halic et al., 2004, Pool et al., 2002). It has been proposed that the displacement allows the translocon to approach and bind the signal sequence thus promoting the release of SRP (Gu et al., 2003, Pool et al., 2002).

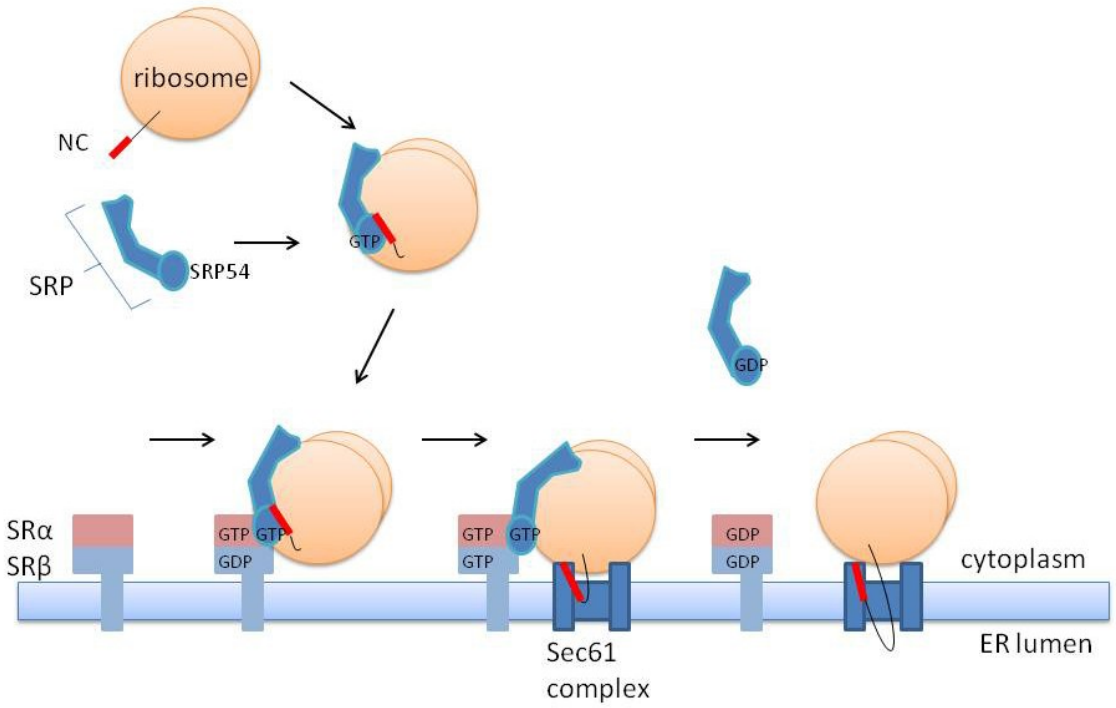


Figure 1.3 SRP targeting of the translating ribosome to the ER membrane. During translation of a nascent chain (NC) containing an N terminal signal sequence SRP binds to the ribosome and the signal sequence which causes translational arrest and the binding of GTP to SRP54. The ribosome is then targeted to the ER where SRα and SRP54 dimerise via their nucleotide binding domains. SRβ is maintained in the GDP bound form via interaction with ribosomal proteins. Upon recruitment of the Sec61p complex SRβ binds GTP and the signal sequence is released from SRP and binds to the Sec61p complex. This process causes hydrolysis of GTP, release of SRP form the ribosome and the SR and the resumption of protein translation.

1.2.3 Targeting to the ER via Post-translational pathways

Most proteins use the co-translational pathway for protein integration, most strikingly in mammalian cells where the post-translational pathway seems less developed than in yeast (Garcia and Walter, 1988). The co-translational pathway provides a protective environment for the nascent protein which helps it to achieve correct topology as translation and integration are coupled. In the post-translational pathway proteins are not protected by the translocon as they emerge from the ribosome but can bind chaperones which aid their solubility (Chirico, 1992, Deshaies et al., 1988). These chaperones are then released before their translocation into the ER (Plath and Rapoport, 2000). The details of these processes remain unclear. In many cases SRP is not required for the post-translational pathways (Ng et al., 1996). However, many post-translational substrates contain a signal sequence which is generally of lower hydrophobicity than for co-translationally targeted substrates (Ng et al., 1996). Even though SRP is not required in the post-translational pathway it has been shown that post-translational substrate pre-pro-α-factor (ppαf) can still bind SRP during translation (Plath and Rapoport, 2000, Ng et al., 1996). In addition, the Nascent chain Associated Complex (NAC) also binds nascent chains in a protective manner (Wang et al., 1995). For example, ppαf is associated to both NAC and SRP during translation.

Following translation, post-translational substrates, such as ppαf, have been shown to bind HSP70 chaperones and the tailless complex polypeptide 1 (TCP1) ring complex/chaperonin containing TCP1 (TRiC/CCT) (Plath and Rapoport, 2000). In yeast, Ydj1p and the Ssa class of HSP70 chaperones have been shown to affect the post-translational import of the post-translational substrate ppαF but not CPY (Becker et al., 1996). In summary, chaperone binding is thought to play a distinct role in protein targeting by keeping substrates in a loosely folded form which allow their post-translational targeting.

Both the post-translational pathway and the co-translational pathway have been shown to use the ER localised transmembrane Sec61 complex (Plath et al., 2004). This complex has been shown to be in association with the Sec62/63 complex (Panzner et al., 1995, Deshaies et al., 1991). The Sec62/63 complex is comprised of Sec62p, Sec63p and two non essential proteins Sec71p (also known as Sec66p) and Sec72p (Panzner et al., 1995, Brodsky and Schekman, 1993). Sec63p, Sec71p and Sec72p are required for the integration of co-translationaly targeted substrates (Jermy et al., 2006, Willer et al.,

2003, Young et al., 2001, Green et al., 1992). However substrates which are targeted to the ER by the post-translational pathway additionally require Sec62p.

The manner in which post-translational substrates are targeted to the Sec61/Sec62/Sec63 complex is still under discussion (Plath et al., 2004, Lyman and Schekman, 1997, Feldheim and Schekman, 1994). However, it seems that post-translational substrates generally require the signal sequence to bind to the Sec62/Sec63 complex as part of the initial stages of translocation in a manner which is independent of ATP. Interestingly it has been reported that the tail anchored protein, Syb2, can be post-translationally targeted to the ER in a manner that requires SRP, SR and GTP (Abell et al., 2004). Tail anchored (TA) proteins do not possess a signal sequence and therefore binding of SRP is thought to occur at the hydrophobic C terminus.

The tail anchored protein Syb2 has been shown to use a post-translational SRP dependent mode of insertion. However, this mode of insertion is not considered to be widespread and constitutes only a complementary pathway for insertion (Abell et al., 2004). In recent years a complex which delivers TA proteins to the ER has been characterised called the GET complex in yeast and is homologous to the TRC40 complex in mammals (Schuldiner et al., 2008). The GET pathway is not essential, but compromising the GET pathway leads to accumulation of tail anchored protein in the cytoplasm. This includes the TA protein Ysy6p (Schuldiner et al., 2008). In yeast Sgt2p binds the TA protein which then binds to the Get4p/Get5p heterodimer (figure 1.4) (Wang et al., 2010). Get4p/Get5p also binds to Get3p, thereby bringing Sgt2p and Get3p into close proximity. The purpose of this is to facilitate the transfer of the TA protein from Sgt2p to Get3p. On a molecular level the transfer from Sgt2p to Get3p of the tail anchored protein is thought to be mediated by the manner in which Get4p/Get5p binds Get3 thereby exposing a hydrophobic groove in the latter (Chartron et al., 2010). Once bound to Get3p the tail anchored protein is targeted to the ER membrane via Get1p and Get2p (Schuldiner et al., 2008). Get1p and Get2p form a membrane complex which mediates the insertion of tail anchored proteins into the ER in an ATP independent manner. It is still unknown how the integration process occurs. It may be that Get1p and Get2p are only required for localising TAs to the ER membrane or they may possess ‘integrase’ activity. Either model is possible as it is believed that unassisted TA protein insertion is not energetically unfavourable and that

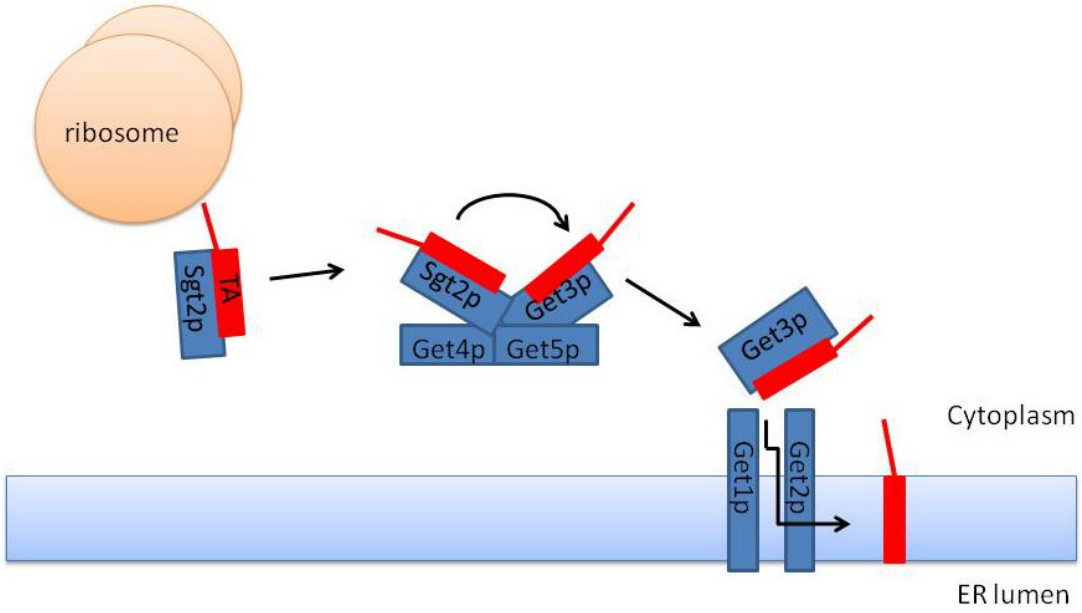


Figure 1.4 Targeting of tail anchored proteins via the GET pathway. Upon completion of translation of a tail anchored protein (TA) Sgt2p associates with its transmembrane domain. The TA is then handed over to Get3p due to binding of Sgt2p and Get3p to Get4p and Get5p. Following handover of the TA from Sgt2p to Get3p the TA protein is targeted by Get3p to Get1p and Get2p which promote insertion of the TA into the ER membrane.

shielding from aggregation, especially for strongly hydrophobic TA proteins, is key to insertion into the ER (Borgese et al., 2007).

1.2.4 Structure of the ER translocon

The ER translocon is composed of a protein heterotrimer which is conserved in structure across eukaryotic as well as prokaryotic organisms (Rapoport et al., 1996). The translocon complex in mammals is called Sec61, Sec61p in yeast and SecY in eubacteria and archaea (Hartmann et al., 1994). The mammalian Sec61 complex is composed of Sec61α, Sec61β and Sec61γ. These are called Sec61p, Sbh1p and Sss1p in yeast, SecY, SecG and SecE in bacteria and SecY, Secβ and SecE in archaea, respectively as shown in table 1.1. In addition, some organisms possess more than one SEC locus (Bensing and Sullam, 2002, Finke et al., 1996). Yeast contains homologs of Sec61p and Sbh1p termed Ssh1p and Sbh2p whereas bacteria contain a homolog of SecY termed SecY2 (Bensing and Sullam, 2002).

The first high resolution structure of the translocon complex was first described for the SecYEβ complex of *Methanococcus jannaschii* (figure 1.5) (Van den Berg et al., 2004). The structure reveals that that SecY contains ten transmembrane (TM) domains which form a pore. TMs 1-5 and TMs 6-10 each form two half rings which are connected by an external loop. The SecE subunit consists of two helices which span the back of the SecY molecule. The SecE molecule lies diagonally across the back of the SecY subunit making contacts with TMs 1, 5, 6 and 10. The SecE subunit is therefore thought to act as a clamp which holds the SecY subunit in the appropriate conformation. Finally, the Secβ subunit is small, consisting of a TM domain and a loop. Secβ makes contacts with the SecY subunit but these are not extensive and Secβ homologs in archaea, mammals and yeast have been shown to be non-essential for the function of the complex (Van den Berg et al., 2004, Bensing and Sullam, 2002, Finke et al., 1996). The SecY subunit forms the core of the SecYEβ complex. Its structure has been described as having an hourglass conformation. The outer surfaces of the translocon contain many charged The SecY subunit forms the core of the SecYEβ complex. Its structure has been described as having an hourglass conformation. The outer surfaces of the translocon contain many charged residues which may stabilise the proteins within the membrane (van den Berg et al., 2004). The interior cavities are lined with uncharged hydrophilic

Table 1.1 Nomenclature of translocon protein homologs

Organism	Name of Complex	Name of complex subunit homolog		
Mammals	Sec61	Secα	Secβ	Secγ
Yeast	Sec61p	Sec61p	Sbh1p	Sss1p
		(Ssh1p)	(Sbh2p)	
Eubacteria	SecYEG	SecY	SecG	SecE
		(SecY2)		
Archaea	SecYEβ	SecY	Secβ	SecE

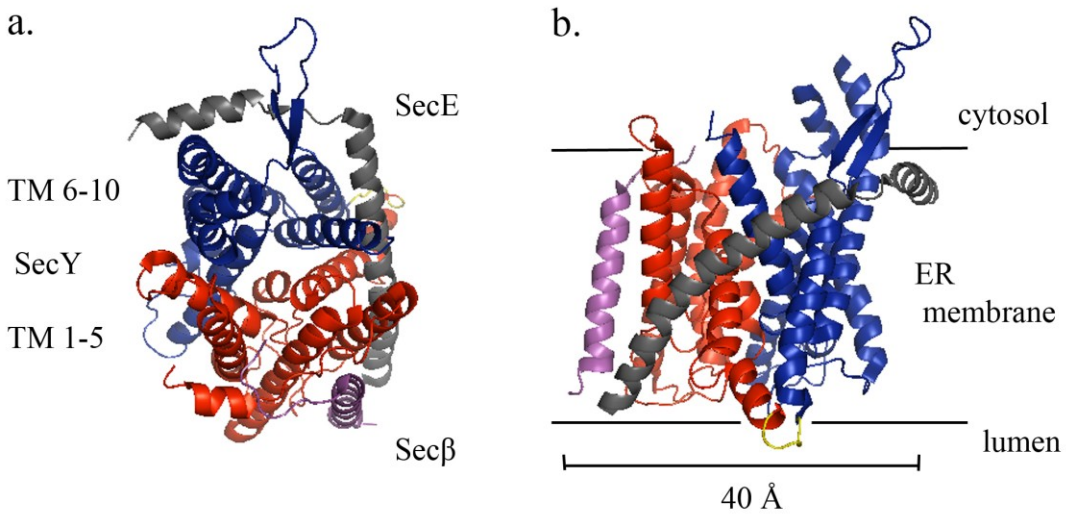


Figure 1.5 X ray Structure of SecYEβ from *Methanococcus jannaschii* at a resolution of 3.2Å. SecYEβ is composed of a heterotrimer of SecE (gray), Secβ (magenta) and SecY (blue and red). SecY forms two half rings composed of TMs 1-5 (red) and TMs 6-10 (blue) which are connected by a loop which protrudes into the lumen (yellow). A view of the complex from the cytosol (a.) and from the lipid bilayer is shown (b). Image made from pdb structure 1RH5 using PyMol and fitted into the membrane as described by Van de Berg et al., (2004).

residues (van den Berg et al., 2004). The two cavities get narrower towards the centre of the pore where there is a narrow pore ring consisting of six hydrophobic residues (figure 1.6 a and b). In the closed conformation the pore is blocked by TM2a of SecY which forms a plug (van den Berg et al., 2004). It is suggested that the main purpose of the plug is to maintain membrane permeability.

1.2.5 The co-translational translocation process

Reconstructed Cryo-EM structures of the mammalian Sec61 complex and the yeast Ssh1p interacting with the actively translating ribosome structure has been solved and been shown to be in good agreement with the structure of SecYEβ previously described (Becker et al., 2009). In addition the Cryo-EM structure of the actively translocating Sec61-ribosome complex was carried out using dog (*Cannis familiaris*) Sec61p complex with plant (*Triticum aestivum*) ribosomes. This further demonstrated the conserved nature of the ribosome-translocon interactions across organisms.

Co-translational translocation starts after binding of the Sec61 complex to the ribosome. This as previously discussed is mediated by the interaction between SRP, the translocon and SR. The binding sites of SRP correspond to points of major contact between the Sec61 complex and the ribosomes (Halic et al., 2006, Halic et al., 2004). This further suggested that ribosome association with the translocon is hindered prior to interaction with SR. This site of SRP, ribosome and translocon interaction has been named the Universal ribosomal Adapter Site and is composed of ribosomal proteins L23a and L35 (L25 and L35 in yeast) (Becker et al., 2009, Halic et al., 2004). The signal sequence is transferred from SRP to the translocon and binds to the translocon in a manner that was first described, in yeast, in the context of post-translational translocation, as binding at the protein lipid interphase (Plath et al., 1998). These results have since been corroborated by structure determination which places the signal sequence as binding to the translocon between TM2b and 7 (figure 1.6 c and d) (Van den Berg et al., 2004). As mentioned previously the Sec61p/SecY complex consists of two connected half ring like structures. TMs 2b and 7 are located on opposite ends of the interconnected two half ring structures but spatially adjacent to each other (Van den Berg et al., 2004).

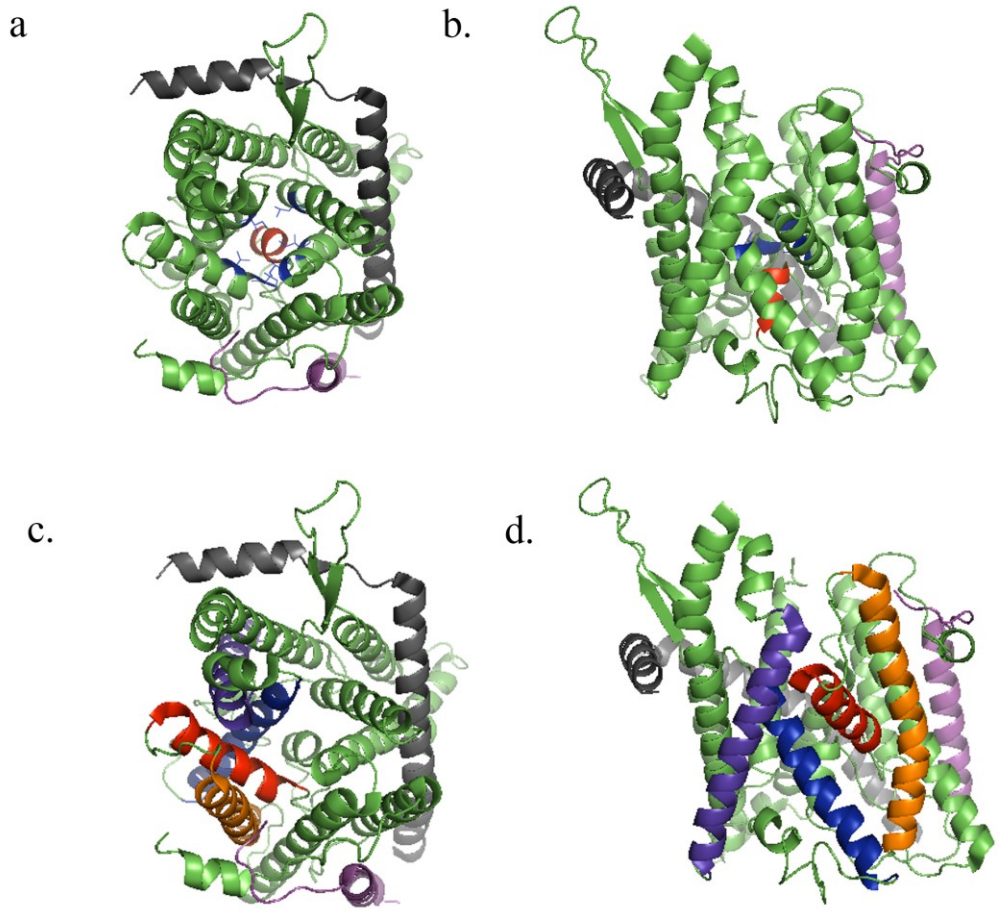


Figure 1.6 Structural features of the SecYEβ complex of *Methanococcus jannaschii*. The pore of SecY (green) is lined by six hydrophobic amino acids (blue) and is occluded in the closed conformation by TM 2a (red) (a and b). During translocation of proteins the signal sequence binds the translocon between TM 2b (red) and TM 7 (blue) (c and d). The lateral gate from which TM segments segregate into the lipid bilayer is composed of TM domains 2b (red), 3 (orange), and 8 (purple). Images made from pdb structure 1RH5 using PyMol.

The Universal Adapter Site is not the only contact point between the translocon and the ribosome and the translocon also makes contacts with ribosomal RNA (Frauenfeld et al., 2011, Becker et al., 2009, Morgan et al., 2002). In addition, early experiments showed that the interaction between the signal sequence, the ribosome and the Sec61p complex to be strong by being resistant to high salt washes (Gorlich et al., 1992b). The dissociation of the ribosome from the translocon requires the release of the nascent by incubation with high salt and puromycin (Gorlich et al., 1992b). In addition, the strong association of the translocon with the ribosome is only maintained in the presence of the nascent chain and therefore in the actively translating ribosome (Jungnickel and Rapoport, 1995).

So far the structure of the translocon has been described as being composed of two half rings which can be plugged by a small transmembrane domain. The question is therefore how this structure mediates the integration of proteins into the ER or their translocation across the ER. The binding of the signal sequence is proposed to trigger rearrangements in the translocon which leads to the displacement of the plug and the opening of a lateral gate into the membrane (figure 1.6 c and d) (Van den Berg et al., 2004). The lateral gate is thought to be the location where TM sequences integrate into the membrane (Van den Berg et al., 2004). The pore, from which the plug is displaced, allows lumenal protein domains to pass through the membrane (Rapoport, 2007). The pore in the closed conformation is narrow, at most, allowing only for segments to pass as sequences devoid of secondary structure (Van den Berg et al., 2004, Tani et al., 1990, Kurzchalia et al., 1988). Van den Berg et al., (2004) however, propose that the pore may widen to allow the passage of an alpha helix. However, further simulations based on this structure suggest that the plug maintains occlusion of the pore thereby guiding hydrophobic segments to the lateral gate but opens in the presence of hydrophilic sequences (Zhang and Miller, 2010). In addition, this process is affected by cross communication between the ribosome and the translocon due to the fact that rpl17 seems to be able to sense the presence of a transmembrane segment and relay this information to the translocon (Lin et al., 2011, Pool, 2009, Liao et al., 1997). The exact mechanism of segregation of hydrophobic and hydrophilic segments is therefore still a point of discussion. However, it seems clear that the lateral gate is the location of TM integration into the ER.

The existence of the lateral gate has lead to the proposal of a "breathing model" for protein integration. In this model the lateral gate, formed by TMs 2b, 3, 7 and 8, allows the nascent chain to sample the hydrophobic interior of the membrane (Rapoport et al., 2004, Van den Berg et al., 2004). Very hydrophobic segments would segregate immediately into the lipid phase whereas more weakly hydrophobic segments would do so more slowly. This model is supported by crosslinking experiments by Ismail et al., (2008) who showed that TM 7 of opsin is released more quickly when replaced by a more hydrophobic TM. It has also been demonstrated that some transmembrane domains exit the translocon and then reassociate with it transiently (Heinrich and Rapoport, 2003, Lin and Addison, 1995, Skach and Lingappa, 1993) It has been suggested that TMs which are unstable, for example due to weak hydrophobicity, need to assemble with a previously synthesised TM in order to exit the translocon (Rapoport et al., 2004). It has been shown that the translcon can support topological inversions of transmembrane domains inside the translocon as this is required for achieving the correct orientation for type II membrane proteins (Devaraneni et al., 2011). One factor that can cause topological inversion is the flanking of a TM with a positive charge. This is known as the positive inside rule (von Heijne, 1989). This positive inside rule can also affect the topology of upstream TM segments (Ojemalm et al., 2012); for example, insertion of a positive charge at the N terminus of a TM can cause an upstream TM domain to mislocalise to the cytosol. Lastly, the translocon has to allow for cytosolic domains to escape into the cytosol. There has been much debate about a potential gap between the ribosome and the translocon (Frauenfeld et al., 2011, Menetret et al., 2000, Crowley et al., 1994, Simon and Blobel, 1991). However, recent publications suggest that the ribosome goes through cycles of tight and loose associations which permit the release of cytosolic domains into the cytosol (Lin et al., 2011, Devaraneni et al., 2011).

1.2.6 The post-translational pathway and accessory factors associated with translocation

The Sec61 complex is sufficient for protein integration at the ER but other proteins are also involved (Gorlich et al., 1992b). However, many of these are lost upon solubilisation of membranes as is the case in Cryo-EM studies (Menetret et al., 2000). In addition, integration or translocation of certain substrates requires additional factors. For example, as previously mentioned, the Sec62/Sec63 complex is required of post-

translational integration (Willer et al., 2003, Panzner et al., 1995, Feldheim and Schekman, 1994, Deshaies et al., 1991). This pathway seems to more prevalent in yeast than mammalian cells as mammalian cells have been said to lack efficient post-translational activity (Meyer et al., 2000). However, this view has recently been challenged by the demonstration that knockdown of the human *SEC62* gene inhibits post-translational transport of proteins (Lang et al., 2012). The way in which the Sec62/63 complex mediates substrate integration is poorly understood. However, it has been shown that the association of the Sec63/Sec62p complex with the Sec61 complex occurs via the brl domain of Sec63p and cytosolic loops of the Sec61 complex (Harada et al., 2011).

The Sec62/Sec63 complex has also been shown to work closely with BiP, known as Kar2p in yeast, and is a HSP70 chaperone (Corsi and Schekman, 1997, Lyman and Schekman, 1997, Rose et al., 1989). Mutations of Kar2p give rise to a protein translocation stalling phenotype. Kar2p binds proteins as they become exposed to the ER lumen (Simons et al., 1995). Kar2p associates with substrate proteins in ATP dependent cycles of binding and release (Hale et al., 2010, Steel et al., 2004). Kar2p has ATPase activity and binds substrate in the ADP bound form. Substrate is released upon exchange of ADP for ATP, a process demonstrated in yeast to be promoted by Lhs1p. Sil1p on the other hand has been shown to promote the ATPase activity of Kar2p. Kar2p has therefore been proposed to function as a molecular ratchet driving protein translocation (Simon et al., 1992). In addition, Kar2p has been proposed to function as a seal for the Sec61p complex thereby maintaining membrane permeability (Haigh and Johnson, 2002). Lastly, it has been reported that the action of mammalian Kar2p, BiP, is coordinated by the Ribosome Associated Membrane Protein (RAMP) ERj1p (Blau et al., 2005, Dudek et al., 2002, Chevalier et al., 2000). This further demonstrates the ability of the ER to coordinate the action of cytosolic and lumenal factors. Further evidence of this coordination has been seen with RAMP4 which has been shown to be recruited to the ribosome translocon complex upon detection by the ribosomal protein L17 of the formation of an alpha helix in the exit tunnel (Pool, 2009). However, the actual function of RAMP4 has still to be elucidated. It is likely that RAMP4 has more than one function as it has been observed to be part of a complex by blue native-PAGE which does not contain the Sec61 complex (Wang and Dobberstein, 1999). Other factors which are known to be recruited to the translocon in mammalian

cells include the TRanslocating chain-Associating Membrane (TRAM) protein and the TRanslocon-Associated Protein (TRAP) complex (Hartmann et al., 1993, Gorlich et al., 1992a). TRAM is required for the translocation of many secreted proteins and is biased for proteins with moderately hydrophobic signal sequences (Voigt et al., 1996). TRAM closely interacts with signal sequences and is proposed to regulate and mediate membrane protein integration (Heinrich et al., 2000, Hegde et al., 1998, High et al., 1993). Similarly, the TRAP complex also associates with nascent chains and the Sec61 complex (Menetret et al., 2005, Mothes et al., 1994). In addition it has been shown to promote integration of substrates which have signal sequences which are thought to poorly interact with the translocon (Fons et al., 2003).

As well as mediating integration other translocon associated factors can modify translocating proteins. These include the Oligosaccharide Transferase Complex (OST) and the signal peptidase complex (SPC) (Silberstein and Gilmore, 1996, Evans et al., 1986). The OST complex and the SPC complex are both translocon associated complexes and conserved across eukaryotes (Wollenberg and Simon, 2004, Gorlich et al., 1992b, YaDeau et al., 1991). The OST catalyses the addition of oligosaccharide chains, also known as N-linked glycosylation, to the nascent polypeptide in the ER lumen. The signal peptidase complex catalyses the removal of the signal sequence in substrate proteins as it emerges from the translcon complex.

The core Sec61 complex as has been discussed here forms a platform for the recruitment of other proteins ranging from the ribosome, in co-translational translocation, to facilitators of translocation such as the TRAM and TRAP complexes and also nascent chain modifiers such as the OST and SPC. Many of these factors have been discovered and continue to be discovered as examplerised by Erj1p but many of these factors remain functionally poorly understood. However, the Sec61p-ribosome complex is clearly not just a ‘pore’ but a highly regulated and modulatable complex which can tailor itself to the needs of each substrate protein being translocated or integrated into the ER.

1.3 Protein integration and translocation in bacteria and plastids

1.3.1 Differences between bacterial and eukaryotic Sec dependent transport

As has previously been discussed in section 1.2 the Sec61 complex is conserved in function and structure across mammals, yeast, eubacteria and archaea (Becker et al., 2009, Van den Berg et al., 2004). In addition, both the post-translational, SRP independent, and the co-translational, SRP dependent, pathways are also conserved. For example, in *E. coli* the ribosome is targeted to the cytoplasmic membrane by a homolog of SRP and an SRP receptor known as FtsY (Pool, 2005). Bacterial SRP is however smaller than eukaryotic SRP and is composed of a single protein, homolog of SRP54, a smaller RNA molecule and lacks an alu domain which in eukaryotes mediates translational arrest. In addition, bacterial SRP does not seem to cause elongation arrest (Raine et al., 2003).

The post-translational pathway in *E. coli* however differs from both yeast and mammalian post-translational translocation. In these eukaryotic systems the protein substrate is thought to be translocated through the Sec61p complex by a Brownian ratchet comprising lumenal Kar2p which binds and releases substrate in an ATP regulated manner (Matlack et al., 1999). However, in bacteria it has been shown that the translocating force is provided by a cytosolic ATPase known as SecA (Kusters and Driessen, 2011). The exact manner in which SecA drives protein integration is poorly understood however it has been shown that SecA binds proteins in an unfolded or partially unfolded state via a clamp domain (Bauer and Rapoport, 2009). This process in some cases is assisted by SecB which maintains proteins in an unfolded state prior to binding to SecA (Bechtluft et al., 2007, Hartl et al., 1990). SecA interacts with SecYEG complex by partial insertion into the latter of a two helix finger and forms contacts with the SecYEG complex which resemble ribosome-SecYEG binding (Kuhn et al., 2011, Erlandson et al., 2008).

1.3.2 Protein transport by the Twin Arginine Targeting pathway

The Twin Arginine Targeting (TAT) pathway transports folded proteins across the bacterial and archeal cytoplasmic membrane as well as across the thylakoid membrane in plant chloroplasts (Albiniak et al., 2012, Frobel et al., 2012, Palmer and Berks, 2012). Many substrates of the TAT pathway contain metal or nucleotide cofactors which

require to be folded in the cytoplasm. In addition, it has been hypothesised that other proteins rely on the TAT pathway for export as they are unable to fold properly in the environment of the periplasm or fold too rapidly for export via the Sec pathway (Albiniak et al., 2012, Palmer and Berks, 2012).

As for the Sec dependent pathway, proteins destined for the TAT pathway contain an N-terminal signal sequence which is composed of an n terminal region which contains a twin arginine motif which is required for efficient transport (Palmer and Berks, 2012, Stanley et al., 2000). This is followed by a moderately hydrophobic region and a C terminal region which contains a basic residue. These features give rise to specificity for entry into the TAT pathway as increasing the hydrophobicity of the hydrophobic region or removal of the basic residue in the C region has been shown to cause entry into the Sec dependent pathway (Cristobal et al., 1999, Bogsch et al., 1997).

Transport via the TAT pathway is largely mediated by three membrane proteins known as TatA, TatB and TatC (Albiniak et al., 2012). TatB and TatC have been shown to recognise and bind the signal sequence. TatC is thought to be involved in the initial binding of the signal sequence by recognition of the twin arginine consensus sequence independently of TatB (Alami et al., 2003). However, TatB also binds the hydrophobic domain of the signal peptide. Together TatB and TatC have been shown to form a tightly associated hetero-oligomer (Bolhuis et al., 2001). In contrast, this same study shows that only a fraction of the TatBC complexes are associated with TatA. Furthermore, it has been shown that TatA is only associated with TatBC when the signal sequence is bound (Mori and Cline, 2002). It is also proposed that TatBC, when substrate bound, promotes the recruitment of TatA in a maner that causes oligmoerisation of the latter (Dabney-Smith and Cline, 2009, Dabney-Smith et al., 2006). TatA is then proposed to form the pore through which the protein is translocated (Gohlke et al., 2005, Oates et al., 2005). Oligomerisation of TatA has been suggested to allow the formation of a pore which is tailored to the size of the protein being translocated thus enabling protein translocation of folded substrates without compromising membrane permeability. This process has been shown to be independent of ATP but instead relies on proton motive force (Bageshwar and Musser, 2007, Yahr and Wickner, 2001, Mould and Robinson, 1991). Following translocation, the signal sequence can be cleaved from the protein and the TatABC complex then disassembles (Luke et al., 2009, Mori and Cline, 2002).

1.3.3 Protein import into chloroplasts by the TIC/TOC complexes

Chloroplasts are composed of an inner and an outer membrane transport through which is largely mediated by two complexes known as the Transport at the Outer membrane of the Chloroplast (TOC) complex and the Transport at the Inner membrane of the Chloroplast (TIC) Complex (Andres et al., 2010, Kovacs-Bogdan et al., 2010). Furthermore, protein import into chloroplasts plays a significant role in chloroplast biogenesis as over 95% of proteins contained in the choloroplast are encoded in the nucleus (Abdallah et al., 2000).

Proteins targeted to the TOC complex contain an N terminal signal sequence which is poorly conserved in sequence (Bruce, 2000). The TOC complex consists of a core complex composed of proteins Toc34, Toc75 and Toc159 (Schleiff et al., 2003b). In addition, other proteins such as Toc64 and Toc12 have been shown to associate with the core Toc complex (Becker et al., 2004, Sohrt and Soll, 2000). Proteins are thought to be targeted to the TOC complex in a manner which is dependent on the phosphorylation state of the signal sequence and involves binding of chaperones in the cytosol and in some cases Toc64 (Andres et al., 2010). For example, Qbadou et al., (2006) have demonstrated that TOC targeted proteins bind to HSP90 which then associate with Toc64. Toc64 is then proposed to bind Toc34 in a GTP dependent manner which is followed by release of the substrate protein in an ATP dependent manner. It has to be noted however that the core TOC complex has been shown to be sufficient for translocation and therefore suggests that cytosolic targeting factors are not essential (Schleiff et al., 2003a). Toc75 is thought to form the pore through which proteins are translocated and to be of a diameter wide enough to allow the passage of only unfolded or partially folded proteins (Hinnah et al., 2002). Schleiff et al., (2003a) propose a model where, after binding of the substrate protein by Toc34, Toc159 drives protein translocation through multiple rounds of GTP hydrolysis. An alternative model however has been proposed where protein translocation may be driven by HSP70 in the intermembrane space (Aronsson and Jarvis, 2008). There is however limited experimental evidence to this effect and the model is largely based on analogy with the mitochondrial HSP70. It is however known that the Hsp70 is recruited to the TOC complex by Toc12 (Becker et al., 2004). In addition, Becker et al., (2004) propose that Hsp70 could maintain substrate proteins unfolded for transfer to the TIC complex by soluble Tic22.

The TIC complex has been shown to comprise eight proteins known as Tic110, Tic62, Tic55, Tic 44, Tic32, Tic 22, Tic21 and Tic20 which work in close association with a stromal HSP93 (Flores-Perez and Jarvis, 2012, Kovacs-Bogdan et al., 2010). To date only Tic110 has been implicated in pore formation of the TIC complex. Tic110 is thought to form the TIC translocon pore on the basis of structure determination by circular dichroism and electrophysiological measurements which suggest that Tic110 forms a six transmembrane cation gated channel which would allow the passage of partially folded proteins (Balsera et al., 2009, Heins et al., 2002). Tic22 and Tic20 have been shown to be able to crosslink to translocating proteins and Tic20 has been proposed to contribute to the formation of the pore (Kouranov et al., 1998, Kouranov and Schnell, 1997). However, Tic22 and Tic20 have been shown only to associate with the Tic110 in the presence of the TOC complex (Kouranov et al., 1998). This is supported by the demonstration that Tic110 can coimmunoprecipitate with Toc75 (Nielsen et al., 1997). It has also been shown that Tic21 is required for inner-membrane translocation (Teng et al., 2006). Interestingly, Tic21 was observed to be associated with a 1 mega Dalton inner membrane complex which is primarily composed of Tic20 and required for protein transport (Kikuchi et al., 2009). Furthermore, this complex was shown not to contain Tic110 which suggests the existence of a secondary pore. It has been suggested that Tic20 may form this secondary pore (Flores-Perez and Jarvis, 2012). However, Kikuchi et al., (2009) suggest that the pore may be formed by as of yet unidentified other components of this 1 mega Dalton complex.

Translocation across the TIC complex is thought to be driven by stromal HSP93 (Chou et al., 2006). In the model proposed by Chou et al., (2006) substrate bound Tic110 binds Tic40. Tic40 then recruits HSP93 which allows the latter to bind and excise the substrate protein in an ATP dependent manner. However, the function of stromal HSP70 has also been shown to function in protein import by the TIC/TOC complexes and to co-immunporecipitate with the latter (Shi and Theg, 2010). In addition, to activation by HSPs the function of the TIC/TOC complex is also regulated by other members of the Tic complex such as Tic62, Tic55 and Tic32 (Kovacs-Bogdan et al., 2010). The TIC/TOC complex therefore imports unfolded or partially folded proteins across the inner and outer chloroplast membranes in an ATP and GTP dependent manner.

1.3.4 Protein transport across mitochondrial membranes

Similarly to chloroplasts, over 95% of mitochondrial proteins are encoded in the nucleus (Becker et al., 2012). Mitochondria are composed of an outer and an inner membrane into or across which proteins need to be transported or integrated and is mediated by several complexes. However, transport at the outer membrane for most, if not all, proteins requires the Translocase of the Outer Membrane (TOM) complex (Becker et al., 2012, Endo et al., 2011, Mokranjac and Neupert, 2009). Integration at the outer membrane is, however, assisted by the Sorting and Assembly Machienry (SAM) complex, also known as the TOB complex. Transport across or into the innermembrane is mediated by Translocases of the Inner Membrane (TIM) such as the TIM22 and TIM23 as well as the OXA1 complex. In addition, translocation across or integration into both membranes is assisted by small TIM proteins.

Proteins destined for mitochondrial import can be classified into two categories: those with and without N-terminal signal sequences (Endo et al., 2011, Brix et al., 1999). These N terminal sequences are poorly conserved in sequence and have a length of between 12 to 70 amino acid residues (Roise and Schatz, 1988). These sequences are recognised by the TOM complex receptor, Tom20. The Tom complex however possesses a second receptor known as Tom70 which recognises proteins without N terminal signal sequences. The recognition of substrates by Tom70 is mediated by cytoslic HSP90 and HSP70 (Young et al., 2003). After recognition by the receptor proteins the substrate protein is transferred to Tom40 for translocation in a manner which is organised by Tom22 (van Wilpe et al., 1999). Tom40, similarly to Toc75, forms a beta-barrel (Hill et al., 1998). The TOM complex pore is of approximately 25Å which is large enough to accommodate partly folded proteins such as a helix-loop-helix domain (Endo et al., 2011). It is currently unknown how translocation across the Tom40 channel is driven however it has been suggested that an electrostatic potential could provide the translocating force (Mahendran et al., 2012).

It has been shown that Tom40 is capable of lateral gating of substrates into the outer membrane (Harner et al., 2011). Other integral proteins, such as beta barrel proteins, do not use this lateral gate and are translocated into the intermembrane space (IMS) where they bind small TIM proteins which is thought to direct them to the SAM complex for their integration (Dukanovic and Rapaport, 2011). For example, it has been demonstrated that small TIM proteins are soluble factors of the IMS deletion or

mutation of which leads to defective beta-barrel protein biogenesis (Wiedemann et al., 2004). As for the TOM complex, partitioning into the lipid phase by lateral gating of beta-barrel proteins from the SAM complex has been proposed but not demonstrated and therefore the mode of insertion of this complex remains elusive (Dukanovic and Rapaport, 2011).

Small TIM proteins also guide proteins to the TIM22 complex for integration or translocation into the inner membrane (Koehler et al., 1998). Initial binding of substrate proteins to the TIM22 complex has been shown to be energy independent which is then followed by energy dependent insertion into the translocon and inner membrane (Rehling et al., 2003, Kovermann et al., 2002). In addition, Tim22 has been shown to form a voltage gated ion channel with a pore size of 11-18Å and the integration of substrate proteins to be powered by the membrane potential.

The inner membrane also contains the TIM23 complex which recognises the N terminal signal sequence of substrate proteins as they emerge from the TOM complex (Mokranjac et al., 2003, Yamamoto et al., 2002, Bauer et al., 1996). Tim23 is proposed to form a 13-24Å channel and translocation of proteins is driven by both the membrane potential and ATP hydrolysis (Truscott et al., 2001, Ungermann et al., 1994, Martin et al., 1991). The membrane potential is thought to translocate the charged N terminal signal sequence which is then followed by an ATP dependent Brownian ratcheting mechanism driven by matrix HSP70 (Liu et al., 2003, Bauer et al., 1996). The TIM23 complex is also able to integrate proteins into the inner membrane, however, membrane proteins can also be translocated and then reinserted in a manner that depends on Oxa1p (Chacinska et al., 2005, Hell et al., 1998). Intrestingly, the Oxa1p is also able to bind ribosomes and implicated in the co-translational translocation of mitochondrial encoded proteins (Szyrach et al., 2003). Lastly, following translocation into the matrix N-terminal signal sequences can be cleaved to yield the mature protein (Mossmann et al., 2012).

1.3.5 Comparison of different membrane protein translocation complexes.

Transport by the Sec, TIC/TOC, TIM/TOM complexes all proposed to translocate proteins which are in at least a partially unfolded state (Endo et al., 2011, Van den Berg et al., 2004, Hinnah et al., 2002). The structures of these pores, although poorly

characterised in many cases, display at least two types of structures which include the beta-barrel pores such as the Toc75 and Tom40 pores or the “hour glass” shaped Sec61 complex formed by helical transmembrane domains (Van den Berg et al., 2004, Hill et al., 1998). However, both types are proposed to mediate both translocation and lateral gating (Harner et al., 2011, Rapoport et al., 2004). The TAT pathway is distinctly different as it translocates fully folded proteins with a pore which is formed by oligomertisation of TatA subunits in a manner which is thought to polymerise a pore tailored in size for the substrate (Frobel et al., 2012). Targeting of substrate proteins to the correct pore is however a conserved feature of protein translocation. This usually takes the form of an N-terminal signal sequence however targeting may also be achieved by internal signal sequences such as TIM22 complex destined proteins (Palmer and Berks, 2012, Kovermann et al., 2002, Bruce, 2000, Roise and Schatz, 1988, von Heijne, 1985). The driving forces of translocation vary. One of the most prominent models for translocation is the Brownian ratchet model which has been proposed for the Sec, TIM and TIC dependent translocation which requires ATP (Chou et al., 2006, Liu et al., 2003, Matlack et al., 1999, Simons et al., 1995). However, the SecA mediated translocation is also proposed to occur via active insertion (Kusters and Driessen, 2011). Other forces which promote translocation are membrane potentials as shown in the function of the TIM22, TAT and also TIM23 dependent pathways (Frobel et al., 2012, Rehling et al., 2003, Truscott et al., 2001). Lastly, it can be noted that import of nuclear encoded proteins via the TOC or TIM complexes occurs post-translationally as ribosomes have not been shown to bind with high affinity to the outer membranes of these plastids (Andres et al., 2010, Mokranjac and Neupert, 2009). In contrast, the Sec machinery is capable of both co-translational and post-translational integration of proteins as previously discussed in sections 1.2.2 to 1.2.6. Protein translocation systems therefore display diversity in their molecular machinery but also show mechanistic similarities.

1.4 Forward trafficking of proteins from the ER

Following integration, proteins that are not ER resident need to be targeted to their correct end cellular localisation. This process is carried out by a system of directed vesicular trafficking. The starting point of forward trafficking from the ER occurs at ER exit sites (ERES) (Okamoto et al., 2012, Shindiapina and Barlowe, 2010). In *S.*

cerevisiae, ERES was identified to be located in areas of the ER with high curvature and that destabilisation of ER curvature domains was shown to cause displacement of ERES and cause changes in Golgi morphology. ERES localisation in the ER is spatially distinct from sites of protein translocation as ribosomes are depleted on the plasma membrane facing side of the ER which contains the high curvature domains associated with ERES (West et al., 2011).

ERES is the location of the formation of COPII vesicles which mediate forward trafficking from the ER to the Golgi (Barlowe et al., 1994). The formation of these vesicles is initiated by Sar1p which upon exchange of GDP for GTP initiates ER tubular membrane deformation (Lee et al., 2005). This leads to the recruitment of a heterodimer composed of Sec23p and Sec24p which exacerbates the tubular deformation initiated by Sar1p by coating the outer surface of the vesicle. This event is then followed by the recruitment of the heterotetramer Sec13/Sec31p which is proposed to stabilise the vesicle (Gillon et al., 2012). Sar1p, Sec23p, Sec24p, Sec13p and Sec31p have been demonstrated to constitute the minimal machinery required for vesicle formation *in vitro* (Figure 1.7) (Matsuoka et al., 1998). However, *in vivo* other factors are required such as Sec12p and Sec16p for ERES formation (Shindiapina and Barlowe, 2010).

Sec23/24p recruitment to the ERES is stabilised by the presence of cargo proteins which are to be trafficked to the Golgi (Forster et al., 2006). Consistent with this is the fact that Sec23/24p has been shown to be able to bind cargo proteins (Miller et al., 2003, Mossessova et al., 2003). The term 'cargo' loosely refers to distinct sets of integral membrane proteins: proteins which are substrates for forward trafficking such pro alpha factor; and Yor1p and proteins which direct forward trafficking such as SNARES (Figure 1.7) (Castillon et al., 2009, Pagant et al., 2007). These proteins may bind directly to Sec23/24p or via adaptor proteins as is the case for Yor1p and pro-alpha factor respectively. Much of the knowledge however surrounding Sec23/24p binding has arisen from the study of SNARE proteins. Two populations of SNARE proteins exist: tethering SNARES (T SNARES); and vesicular SNARES (V SNARES) the expressions of which define the targeting route (Nichols and Pelham, 1998). Three independent cargo binding sites have been located on Sec24p which have been since named A, B and C (Buchanan et al., 2010). The A and B sites are well characterised with the consensus binding sequences to have been established to be YxxxNPN and

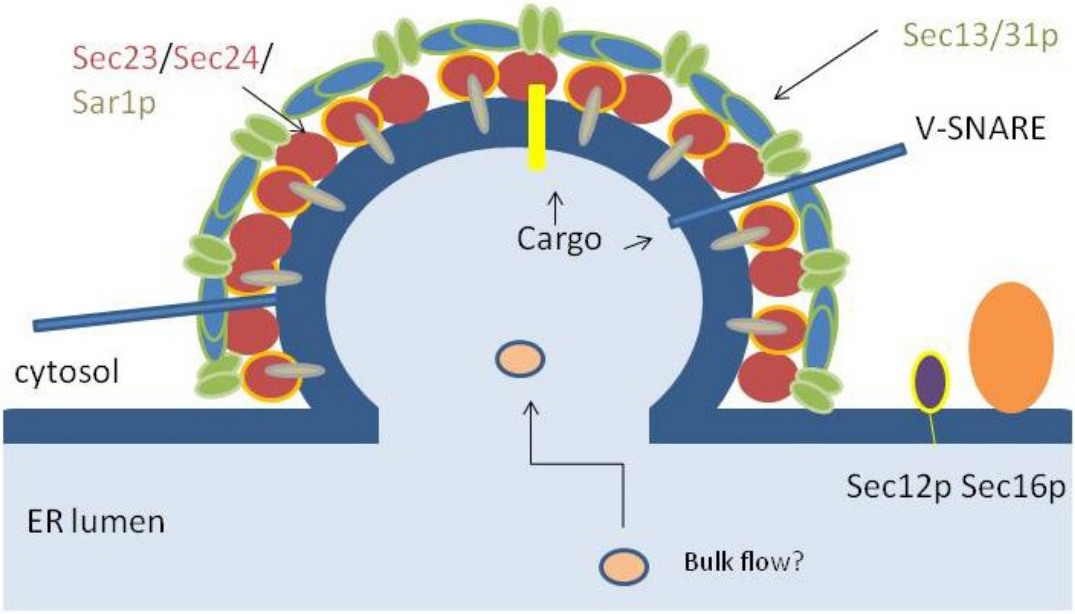


Figure 1.7 Formation of vesicles which mediate forward trafficking from the ER Recruitment of Sar1p to the Sec23/24p heterodimer initiates deformation of the ER membrane and recruitment of Sec13/31p. This process is also known to require Sec12p and Sec16p. The Sec23/24p contains multiple binding sites which bind cargo proteins such as proteins destined for export such as Yor1p and V-SNARES which direct vesicles to the appropriate docking sites. It is thought that proteins may also be transported without interaction with Sec23/24p and is known as bulk flow.

LxxME/LE or DxE respectively (Mossessova et al., 2003, Votsmeier and Gallwitz, 2001, Nishimura et al., 1999). The C site has been identified in complex with a peptide and therefore no consensus sequence has been constructed as of yet for this site (Miller et al., 2003). However, this highlights the importance of binding motifs. For example, Yor1p has been shown to contain a functional DxE motif (Pagant et al., 2007).

It is however proposed that not all substrates require a specific interaction with the COPII proteins. One of the mechanisms by which this would occur is termed 'bulk flow' (Thor et al., 2009, Wieland et al., 1987). Thor *et al.,* (2009) propose that bulk flow works as a form of passive packaging of substrate proteins into COPII vesicles. Furthermore, that 'bulk flow' movements of proteins may be restricted by association with quality control chaperones, such as calnexin, until the protein has reached the appropriate folding state for export.

There is currently a lack of evidence surrounding bulk flow theory. However, there is evidence that Sec24p is not universally required (Fatal et al., 2004, Kurihara et al., 2000). For example, it has been demonstrated that the glycoprotein Hsp150p is trafficked in the absence of Sec24p or a homolog of Sec24p, Lss1p (Fatal et al., 2004, Kurihara et al., 2000). However, there is a second homolog of Sec24p known as Lst1p (Roberg et al., 1999). It is clear therefore that more knowledge of COPII vesicle trafficking is required before it is known whether 'bulk flow' plays a *bona fide* role in forward trafficking.

1.5 ER associated degradation (ERAD)

Following targeting to the ER targeted proteins are subject to quality control mechanisms which sense their folding state (Benyair et al., 2011, Vembar and Brodsky, 2008). Proteins which are recognised as mis-folded or modified can interact with chaperones which facilitate achieving the correct confirmation. Proteins which fail to achieve a stable conformation can then be targeted for degradation by the ER associated degradation machinery (ERAD). This process leads to the ubiquitination, retrotranslocation out of the ER and degradation by the proteasome (Bagola et al., 2011, Finley, 2009).

1.5.1 Ubiquitin ligases form the core of the ERAD machinery

A central process of the ERAD machinery is ubiquitination of substrate proteins. This process is carried out by ubiquitin ligases. Ubiquitin is encoded by a family of genes (Ozkaynak et al., 1987). Many of these genes however encode fusions of ribosomal protein to ubiquitin and the ubiquitin moiety has been shown to function in ribosome biogenesis (Finley et al., 1989). The only gene encoding ubiquitin alone is *UBI4* which encodes polyubiquitin and is then cleaved to monomers after translation to yield monoubiquitin (Ozkaynak et al., 1987). Ubiquitin is added to substrates by a cascade of events mediated by E1, E2 and E3 ligases (Haas and Siepmann, 1997). The yeast E1 ligase is encoded by the Ubiquitin Activating enzyme 1, *UBA1* (McGrath et al., 1991). E1 enzymes function by binding ubiquitin in a two step process which includes ATP dependent adenylation of ubiquitin which promotes transfer of ubiquitin to the active site of the E1 ligase thus presenting ubiquitin in a competent form for transfer to E2 ligases (Pickart et al., 1994). In yeast, E2 ligases involved in ERAD, are the Ubc1p, Ubc6p and the Ubc7p (Chen et al., 1993, Seufert et al., 1990). The E3 ligases include Doa10p and Hrd1p (Bays et al., 2001a, Swanson et al., 2001).

Doa10p and Hrd1p form the core of two distinct ERAD degradation pathways (Carvalho et al., 2006, Vashist and Ng, 2004). This has been largely demonstrated on the basis of substrate degradation requirements. For example the lumenal protein CPY* is a constitutively unstable protein which requires Hrd1p but not Doa10p for its degradation (Swanson et al., 2001, Vashist and Ng, 2004). The constitutively unstable polytopic membrane protein Hmg2p which can be stabilised by point mutations in its transmembrane domain is also a Hrd1p substrate (Theesfeld et al., 2011, Hampton et al., 1996). However proteins such as Ste6-166 that contain a mutation in their cytosolic domain are degraded by the Doa10p pathway (Huyer et al., 2004, Vashist and Ng, 2004). The Doa10p dependent pathway has been shown to be biased for the degradation of integral ER membrane substrates with misfolded cytosolic domains. The Hrd1p dependent pathway on the other hand seems to exert a preference for the degradation of substrates with unstable transmembrane or lumenal domains. These observations have lead to a classification of ERAD pathways termed ERAD-C (cytosolic), ERAD-M (membrane) and ERAD-L (lumenal) (Figure 1.8) (Carvalho et al., 2006). However, proteins which have both misfolded cytosolic and transmembrane or lumenal sequences may be degraded by either Hrd1p or Doa10p complexes (Vashist

and Ng, 2004). One such protein is the clinically important human Cystic Fibrosis Transmembrane conductance Regulator (CFTR) mutant forms of which cause cystic fibrosis (Gnann et al., 2004).

1.5.2 Recognition of ERAD substrates

It is currently proposed that both Doa10p and Hrd1p can directly recognise misfolded substrates (Sato et al., 2009, Ravid et al., 2006). The rational for this relies on the fact that genetic interaction screens demonstrated that only the E2 ligases and Doa10p are required for Doa10p dependent degradation (Ravid et al., 2006). The evidence for direct substrate recognition by Hrd1p was described by point mutations of transmembrane domains in Hrd1p which was shown to lead to defective degradation but not association of Hrd1p with Hmg2p (Sato et al., 2009). On this basis the authors suggest a "hydrophilic scanning" model for Hrd1p ERAD-M substrate recognition where proteins become substrates for Hrd1p dependent ERAD upon exposure of normally buried hydrophilic residues.

Even though Hrd1p and Doa10p are required and proposed to be sufficient for ERAD substrate recognition it is becoming clear that additional recognition factors play an important role for the degradation of certain substrates. For example the Doa10p dependent pathway has been shown to require *SSA* gene family members for efficient degradation of the ERAD-C substrate Ste6-166 (Han et al., 2007). The *SSA* genes encode HSP70 cytosolic chaperones which bind substrate proteins promoting their folding and preventing their aggregation (Bukau and Horwich, 1998, Hottiger et al., 1992). Further evidence of the involvement of HSP70 chaperones in ERAD has been observed for the Hrd1p dependent pathway where Kar2p has been shown to be required for maintaining CPY* in an ERAD competent state by preventing aggregation under conditions where the Unfolded Protein Response (UPR) is induced due to temperature stress (Nishikawa et al., 2001).

In addition to chaperones promoting ERAD Hrd1p, unlike Doa10p, has been shown to form complexes with many other proteins involved in substrate recognition (Carvalho et al., 2006). The most notable of these is Hrd3p which is in a stoichometric complex with Hrd1p (Gardner et al., 2000). Hrd3p has been shown to stabilise Hrd1p and

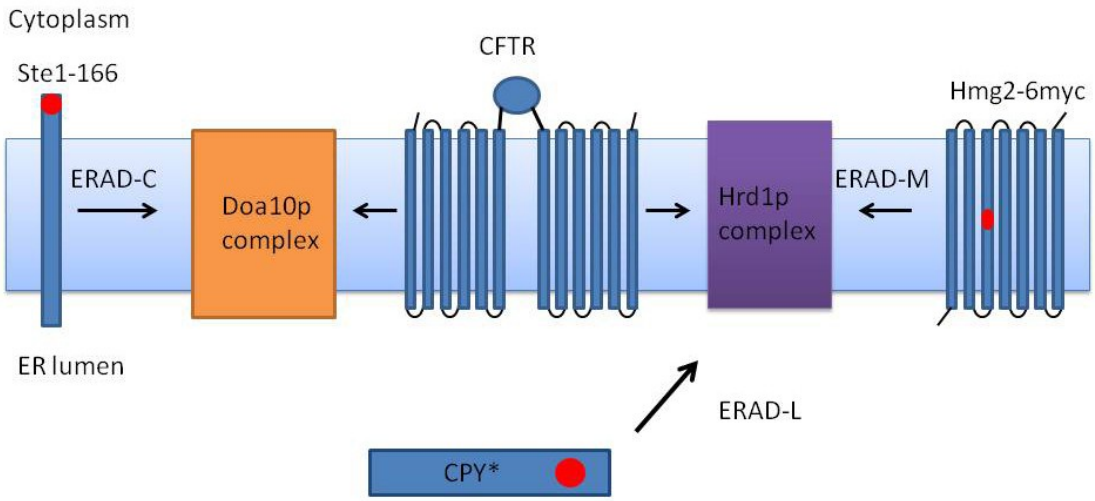


Figure 1.8 ERAD pathways. The Doa10p and Hrd1p complexes constitute two distinct pathways of ER associated degradation. Proteins with lesions (red dots) in their cytosolic domains are degraded by the Doa10p complex in a pathway termed ERAD-C. Proteins with lesions in their transmembrane domains such as Hmg2-6myc or lumenal domains such as CPY* are degraded by the Hrd1p complex in pathways termed ERAD-M and ERAD-L respectively. Some proteins such as Cystic Fibrosis Transmembrane conductance Regulator (CFTR) can be degraded by either the Doa10p or Hrd1p pathway.

hypothesised to promote interaction of Hrd1p with ERAD substrates. In addition, it was shown that overexpression of Hrd1p could partially rescue the ERAD defect in a *Δhrd3* strain. Hrd3p was later shown to form a complex with Kar2p and Yos9p by coimmunoprecipitation (Denic et al., 2006). Hrd3p and Yos9p are suggested to form a gating complex for the Hrd1p dependent pathway. This model relies on the basis that, firstly, deletion of *YOS9* or *HRD3* caused a block in degradation of CPY* but in a strain where Hrd1p expression was upregulated deletion of *YOS9* had no effect on ERAD and deletion of *HRD3* alleviated the ERAD defect. Secondly, that when the glycosylation sites of CPY* were abolished by mutation CPY* became stable in *WT* cells but degraded in a *Δhrd3* strain overexpressing Hrd1p. This therefore showed that Yos9p and Hrd3p regulate ERAD substrate specificity and inhibit Hrd1p from erroneously targeting substrates for degradation.

The manner in which Yos9p regulates substrate specificity has been well documented in the recognition of glycoslyated proteins (Quan et al., 2008, Denic et al., 2006, Bhamidipati et al., 2005, Kim et al., 2005, Szathmary et al., 2005, Buschhorn et al., 2004). Protein glycosylation occurs in the ER by the Oligossacharride Transferase Complex (OST) in a process which tightly associated with protein translocation (Scheper et al., 2003, Knauer and Lehle, 1999). In this process the N-linked glycan $Glc_1Man_9GlcNAc_2$ is added at consensus sequence Asn-xxx-Ser/Thr. The glycan is then sequentially trimmed until the protein is no longer recognised as misfolded and exported or is targeted for degradation after the end of the trimming sequence (Benyair et al., 2011). The end of the trimming sequence is mediated in yeast by Htm1p which catalyses the removal of a mannose residue (Clerc et al., 2009). Yos9p has been demonstrated to preferentially recognise terminally trimmed glycans and that deletion of upstream glycan trimming enzymes negatively impacts ERAD of CPY* (Quan et al., 2008). On the basis of this evidence the authors suggest a model where Yos9p mediates selection of glycosylated substrates thereby further implicating it in a gating mechanism for Hrd1p dependent pathway. It has to be noted however that Yos9p substrate recognition does not depend on a proteins glycosylation state as CPY* with mutated glycosylation sites is still recognised by Yos9p (Bhamidipati et al., 2005). This therefore suggests that Yos9p recognises a bipartite signal and has lead to suggestions that Yos9p's function may be extended to non-glycosylated substrates (Jaenicke et al., 2011, Benitez et al., 2011). However, this assertion is questioned by the

fact that no native unglycosylated ERAD substrates have yet been reported which require Yos9p for efficient degradation.

In addition to Yos9p and Hrd3p, other proteins have been associated with the Hrd1p complex and include Der1p and Usa1p. Usa1p functions as a scaffold protein which promotes oligomerisation of the Hrd1p complex which has been shown to be a prerequisite for degradation of membrane proteins (Horn et al., 2009). In addition, Usa1p has been shown to promote recruitment of Der1p to the Hrd1p complex which has been shown to be required for efficient degradation of ERAD-L substrates such as CPY* (Hitt and Wolf, 2004, Taxis et al., 2003, Knop et al., 1996).

1.5.3 Retrotranslocation, ubiquitination and degradation of ERAD substrates.

In order for the Doa10p and Hrd1p E3 ligases to mediate substrate ubiquitination they require the activity of the E2 enzymes (Bays et al., 2001a, Chen et al., 1993). The Doa10p and Hrd1p proteins differ in their requirements for E2 enzymes. The Doa10p dependent pathway requires Ubc6p and Ubc7p whereas the Hrd1p dependent pathway requires Ubc7p and Ubc1p (Bays et al., 2001a, Swanson et al., 2001, Chen et al., 1993). The function of the cytosolic protein Ubc7p however is dependent on Cue1p for its recruitment to the ER membrane (Biederer et al., 1997). It is currently not well understood how E3s catalyse the transfer of ubiquitin from E2 to the substrate protein but it is proposed that the E2 enzyme associates with the E3 enzyme via the E3 RING H2 domain in a manner which promotes transfer of ubiquitin (Deshaies and Joazeiro, 2009). RING H2 domains, which both Doa10p and Hrd1p possess, are characterised by a conserved motif which includes conserved histidine and cysteine residues (Swanson et al., 2001, Bordallo et al., 1998, Freemont, 1993).

The E3 and E2 ligases catalyse the ubiquitination of substrate proteins which promotes their degradation by the 26S proteasome (Deshaies and Joazeiro, 2009). It has been shown that lysine residues of ubiquitin bind to the substrate protein which when modified lead to stabilisation of ERAD substrates (Shang et al., 2005). In addition, ubiquitin addition to substrates results in a polyubiquitin chain being attached to substrates the length of which has been shown to impact efficiency of degradation (Thrower et al., 2000, Chau et al., 1989). Ubiquitin has been proposed to directly promote protein unfolding (Hagai and Levy, 2010). However, ubiquitination of

substrates is also associated with the recruitment of factors thought to drive protein excision from the ER membrane and targeting to the proteasome (Raasi and Wolf, 2007). One such factor is the cytosolic AAA ATPase, Cdc48p, conditional mutants of which resulted in defective degradation of Hmg2-6myc and CPY* at restrictive temperature (Rabinovich et al., 2002). In addition Cdc48p was also shown to be in a complex with other cytosolic proteins Npl14p and Ufd1p (Hitchcock et al., 2001). Mutations of the latter two proteins were also shown to cause defects in ERAD (Jarosch et al., 2002). The cytosolic Cdc48-Npl4-Ufd1p has also been shown to be recruited to the Hrd1p and Doa10p complexes via the transmembrane protein Ubx2p therefore allowing interaction with the ERAD substrate (Schuberth and Buchberger, 2005). Together these proteins have been proposed to function as a molecular ratchet which actively excises proteins from the ER (Raasi and Wolf, 2007). Upon excision from the membrane proteins are targeted to the proteasome where they are digested in a process which is proposed to be mediated by factors including Rad23p, Dsk2p and Rpn10p (Richly et al., 2005, Verma et al., 2004, Elsasser et al., 2004, Elsasser et al., 2002, Chen and Madura, 2002). Richly et al., (2005) propose a model where Rpn10p and Rad23/Dsk2p are distinct proteasome targeting factors. In this model Rad23p and Dsk2p can selectively bind ubiquitin chains which have been modified by Cdc48p associated proteins. Rpn10p has been proposed to form part of alternative degradation pathway which targets polyubiquitinated substrates which have escaped regulated processing by the Cdc48p complex or associated factors. Either pathway however culminates in deubiquitantion of substrates and degradation by the proteasome (Finley, 2009).

One area which has still not been elucidated is how the E3 ligase complexes mediate retrotranslocation of proteins across the ER (Bagola et al., 2011). Multiple studies have suggested a role for the Sec61p complex in retrotranslocation of ERAD substrates (Schafer and Wolf, 2009, Willer et al., 2008, Zhou and Schekman, 1999, Pilon et al., 1997, Plemper et al., 1997, Wiertz et al., 1996). From these studies it is clear that CPY*, and membrane bound derivatives thereof, require functional Sec61p complex for efficient ERAD-L (Willer et al., 2008, Plemper et al., 1997). These observations were then supported by the observation that Sec61p coimmunoprecipitated with glycosylated CPY* and the Hrd1p complex (Schafer and Wolf, 2009). However, photocrosslinking experiments have failed to identify an association of ERAD substrates to Sec61p

(Carvalho et al., 2010). It is also believed that Sec61p association with ERAD may be indirect and the result of ERAD substrates remaining bound to the Sec61p translocon following translocation (Bagola et al., 2011). However, it has also been shown that Sec61p bind to the proteasome thereby further supporting involvement of Sec61p in ERAD (Kalies et al., 2005). It is therefore clear that the Sec61p complex plays a role in ERAD but the extent of this role is still unknown.

1.6 The unfolded protein response

Living organisms have evolved to survive in a dynamic environment which include changes in temperature, nutrient availability and chemical toxicity (Gasch, 2003). These conditions can cause improper folding of proteins and therefore perturb homeostasis which can lead to cell death. In the yeast ER it has been shown that accumulation of unfolded proteins leads to the activation of a pathway known as the Unfolded Protein Response (UPR) which causes the transcription of factors which assist protein folding (Cox et al., 1993, Mori et al., 1993, Mori et al., 1992). Compromising this pathway results in cell death under conditions of stress (Cox et al., 1993).

In yeast the level of unfolded proteins is sensed by a transmembrane protein known as Ire1p (Cox et al., 1993, Mori et al., 1993). Ire1p is a type I ER resident transmembrane protein with a lumenal N terminal domain which senses the folding state of lumenal proteins whereas the cytosolic domain contains an RNase domain and a protein kinase domain (Sidrauski and Walter, 1997, Cox et al., 1993, Mori et al., 1993). The N terminal sequence of Ire1p has been shown to directly bind to the constitutively misfolded CPY* protein (Gardner and Walter, 2011). Furthermore, it was shown that CPY* binding to Ire1p caused induction of the UPR. The binding of Ire1p to the substrate is proposed to be analogous to that of the Major Histocompatibility Complex (MHC) which characterised by a deep groove (Credle et al., 2005). Furthermore the binding of Ire1p to substrates is proposed to mediate dimerisation or oligomerisation of Ire1p and transphosphorylation of the protein kinase domains (Credle et al., 2005, Shamu and Walter, 1996). This model also rationalises the dominant negative effect observed when coexpressing *WT* and C terminally truncated Ire1p (Shamu and Walter, 1996).

The process of detecting unfolded proteins in the ER has also been proposed to not only involve Ire1p but also Kar2p. Kar2p was initially implicated in the UPR response as, contrary to Ire1p, its transcription is elevated in cells with increased levels of unfolded proteins (Mori et al., 1993, Rose et al., 1989). Kar2p expression levels were then shown to be upregulated by the UPR (Cox et al., 1993). Kar2p overexpression has been shown to attenuate the UPR (Okamura et al., 2000). However, as has been previously discussed Kar2p has been shown to be involved in post-translational translocation and to be a folding chaperone (Simons et al., 1995). Therefore the effect of overexpression of Kar2p on the UPR could be indirect. Strikingly though Kar2p has been demonstrated to coimmunopreciptate with Ire1p and that induction of the UPR by tunicamycin causes a loss of this association (Okamura et al., 2000). On the basis of these observations Okamura et al., (2000) proposed a model where Ire1p and unfolded proteins compete for Kar2p binding and therefore in conditions with elevated unfolded proteins Kar2p dissociates from Ire1p allowing dimerisation of Ire1p and the induction of the UPR. This model is supported by the observation that Kar2p mutants constitutively bound to Ire1p fail to induce the UPR under conditions of ER stress whereas mutants which poorly associate with Ire1p caused induction of the UPR even in the absence of tunicamycin (Kimata et al., 2003) However, subsequent work suggested that release of Kar2p is not the primary determinant in UPR activation and that activation of the UPR requires interaction of Ire1p with unfolded proteins (Kimata et al., 2007, Kimata et al., 2004, Kimata et al., 2003). Kar2p is therefore involved in UPR induction in a manner which is currently not well understood.

Following Ire1p dependent recognition of unfolded substrates, Ire1p molecules interact via their lumenal domains which allows transphosphorylation by the cytosolic kinase domains (Zhou et al., 2006, Shamu and Walter, 1996). This transphosphorylation is proposed to stabilise and activate the RNase domain of Ire1p (Korennykh et al., 2009, Lee et al., 2008). The RNase domain's function is to splice *HAC1* mRNA (Sidrauski and Walter, 1997, Cox and Walter, 1996). Splicing causes removal of an intron from *HAC1*, referred to as $HAC1^{u}$ (uninduced), to yield $HAC1^{i}$ (induced). The fragments are religated by a tRNA ligase (Sidrauski et al., 1996). Even though both forms of *HAC1* mRNA have been shown to be transported out of the nucleus only $HAC1^{i}$ can be translated into Hac1p (Chapman and Walter, 1997). Hac1p is a transcription factor which binds to promoter sequences containing the Unfolded Protein Response Element

(UPRE) (Cox and Walter, 1996, Mori et al., 1992). *HAC1*^{u} has been shown to adopt base pairing between its 5' UTR and its intron which diminishes ribosome association (Ruegsegger et al., 2001). However, a pool of *HAC1*^{u} mRNA is still associated with translationally stalled ribosomes. This observation lead to the proposal by Ruegsegger et al., (2001) that *HAC1*^{u} mRNA occurs on translationally stalled ribosomes. However, this hypothesis is challenged by the fact that religation of *HAC1* RNA fragments generated require a nuclear localised tRNA ligase, Trl1p (Sidrauski et al., 1996, Clark and Abelson, 1987). Ruegsegger et al., (2001) rationalise this by suggesting that a small pool of the tRNA ligase may localise to the cytosol at endogenous levels as has been observed in the case of overexpression. This hypothesis is supported by more recent data which demonstrated that a small proportion of Trl1p is ribosome associated (Mori et al., 2010).

Induction of the UPR by Ire1p has been described as functioning as a molecular switch which triggers HAC1 mRNA splicing and induction of transcription of UPRE containing genes (Leber et al., 2004). However, evidence is mounting that additional factors affect the UPR. Ypt1p for example has been shown to modulate the UPR by attenuating the UPR following removal of agents causing induction of the UPR (Tsvetanova et al., 2012). The mechanism of action of Ypt1p has been rationalised by the demonstration that Ypt1p promoted the decay of HAC1^{u} mRNA by binding under conditions where the UPR is not induced. Interestingly, Ypt1p has previously been demonstrated to be involved in ER to golgi trafficking thus demonstrating a link between UPR induction and trafficking (Grosshans et al., 2006). It has been shown that both *HAC1*^{u} and *HAC1*^{i} mRNA abundance is increased in cells defective in components of the secretory pathway (Leber et al., 2004). It was shown that this upregulation of *HAC1* mRNA was independent of both Ire1p and the heat shock response but transcriptionally induced by an unidentified factor. This increase in *HAC1* mRNA was then shown to increase levels of Hac1p proportionally thereby identifying a new pathway which the authors call the Super-UPR. In order to determine the significance of induction of the S-UPR pathway gene transcription was investigated by mRNA microarray profiling. This demonstrated that UPR induced genes can be categorised into three classes depending on their degree of induction. Class I substrates showed no difference in induction between activation of the UPR or S-UPR. Class 2 substrates are induced to a 2 to 4 fold greater extent under conditions causing the S-

UPR relative to conditions inducing the UPR alone. Finally, class 3 substrates are induced 4 fold or more. Interestingly, this also lead to functional characterization of these various classes of substrates. Class I substrates included genes encoding ER chaperones and disulphide remodelling proteins such as Kar2p and Pdip (Laboissiere et al., 1995, Rose et al., 1989). Class II substrates included Yip3p involved in ER to golgi transport and class III substrates included Der1p, and Ino1p which are involved in ERAD and membrane biogenesis respectively (Otte et al., 2001, Knop et al., 1996, Hirsch and Henry, 1986). This also demonstrates that the UPR has a wide range of effects which mediate adaptation to ER stress. To assess how widespread the effect of the UPR has on cellular remodelling Travers et al., (2000) used DNA microarray analysis of cellular mRNA. This study demonstrated that the UPR mediates upregulation of proteins involved in a wide range of processes including protein integration, protein folding, ERAD, trafficking, glycosylation and cell wall biogenesis. The UPR is therefore a survival mechanism which is initiated at the ER but initiates widespread cellular remodelling.

1.7 Candidates for involvement in secretory processes

1.7.1 Ysy6p

Ysy6p was first implicated in protein secretion by functional screening of suppressors for defective translocation in *E. coli* (Sakaguchi et al., 1991). It was shown that expression of Ysy6p from *S. cerevisiae* enabled growth of temperature sensitive mutants of SecY at restrictive temperature and improved the translocation of an outer membrane protein of *E. coli*, OmpA. Ysy6p is a short, 7.4 kDa, tail anchored protein which has been shown to be inserted into the ER via the GET pathway (Schuldiner et al., 2008). An investigation into the level of GFP expression, controlled by the UPRE, showed that deletion of *YSY6* moderately induced the UPR. This suggests that deletion of *YSY6* causes the exposure of unfolded proteins, in the ER, to the UPR machinery (Jonikas et al., 2009). This induction of the UPR can be caused by defective protein integration or other downstream factors associated with the secretory pathway (Jonikas et al., 2009). This highthrouput study in addition to demonstrating an interaction with the UPR and Ire1p also demonstrated a genetic interaction with the lumenal chaperone involved in protein folding Lhs1p and Der1p which is required for ERAD-L (Sato and Hampton, 2006, Hitt and Wolf, 2004, Craven et al., 1996). An additional, genetic

interaction was observed with Wbp1p, which is a subunit of the OST complex (Costanzo et al., 2010, Kelleher and Gilmore, 1994). Strikingly, however, the only reported physical interaction of Ysy6p is with Sec62p which is involved in protein integration and translocation across the ER (Yu et al., 2008, Rothblatt et al., 1989). This therefore suggests a direct interaction of Ysy6p with the protein integration machinery. Taken together the data suggest that Ysy6p is involved in secretory processes.

The biochemical function of Ysy6p has however only been poorly studied. Much more data exists concerning the putative mammalian homologs of Ysy6p, RAMP4 also known as SERP1 (Schroder et al., 1999, Yamaguchi et al., 1999). RAMP4 was first identified as a protein which cosedimented with ribosomes under conditions of high ionic strength (Görlich and Rapoport, 1993). It was also observed that after removal of ribosomes by treatment with puromycin that a small proportion (4% of the ribosome associated pool of RAMP4) could be copurified by ion exchange chromatography with the Sec61 complex. This therefore strongly suggested that RAMP4 had a function in processes concerning protein biogenesis. It was later shown that RAMP4 could be crosslinked to the glycosylated type II transmembrane protein Ii (Schroder et al., 1999). Crosslinking of RAMP4 to Ii was efficient as up to 40% of Ii translation intermediates could be crosslinked to RAMP4. Crosslinks were observed from the point at which a lumenal hydrophobic domain is being translocated across the Sec61p translocon and it was also shown that this hydrophobic domain was required for crosslinking to RAMP4. It was also observed that this sequence lay immediately upstream of Ii glycosylation sites. Although the glycosylation sites were not required for crosslinking the authors suggested a model where RAMP4 can recognise hydrophobic sequences in the Sec61 translocon to promote glycosylation of downstream sequences. In addition to being crosslinked to a translocation substrate RAMP4 has been shown to crosslink and coimmunoprecipitate with Sec61β (Yamaguchi et al., 1999). Consistent with this data is the demonstration that the ribosomal protein rpl17 can be crosslinked to sec61β and RAMP4 thereby further suggesting that RAMP4 and Sec61β are in close proximity (Pool, 2009). Interestingly, it was demonstrated that RAMP4 is recruited to the ribosome when the ribosomal protein rpl7 senses the emergence of a transmembrane domain in the ribosomal exit tunnel (Pool, 2009). Together the data from these studies

provide strong evidence that RAMP4 is associated with the translocon and that hydrophobic domains influence recruitment of RAMP4.

RAMP4 has also been implicated in an undefined process which protects proteins from degradation (Yamaguchi et al., 1999). Following chemical induction of the UPR it was shown that the glycosylated transmembrane proteins RAGE and CD8 were degraded. However, surprisingly, overexpression of RAMP4 prevented degradation of these proteins. In addition, it was demonstrated that RAMP4 could be coimmunonprecitated with the chaperone calnexin which promotes protein folding (Lederkremer, 2009). The authors therefore suggest that RAMP4 promotes folding of proteins thereby shielding them from degradation. Again this further implicates RAMP4 with protein biogenesis.

The mRNA transcript levels of RAMP4 were determined in various tissues and found to be highly expressed in tissues with high secretory activity such as the pancreas, prostate and salivary glands in mice and human tissue (Hori et al., 2006). It was also shown that tissues with upregulated RAMP4 correlated with upregulation of members of the Sec61 complex. In addition, it was shown that RAMP4 is important in pancreatic function as glucose tolerance was reduced (Hori et al., 2006). It was shown that deletion of RAMP4 caused an increase in blood glucose concentration relative to *WT* mice. It was also shown that RAMP4-/- pancreatic cells showed a delay in proinsulin biosynthesis upon cells being subjected to increased glucose concentrations. Interestingly, this same study showed that the ER chaperones GRP94 and BIP, which are markers for stress and demonstrated to be involved in ERAD, were upregulated in RAMP4-/- cells (Christianson et al., 2008, Ramakrishnan et al., 1997). The data therefore further implicate RAMP4 in protein biogenesis and ERAD. Lastly, the physiological importance of RAMP4 has been demonstrated in mouse development. RAMP4-/- mice show increased mortality and decreased body size by 3 weeks after birth. Mice which survived then recovered body weight by 12 weeks.

There is therefore strong evidence that RAMP4 has a role in secretory processes such as protein biogenesis and ERAD. The evidence for Ysy6p involvement is however limited. Having said this Ysy6p shows genetic and physical interactions with similar processes as RAMP4 notably, protein integration, ERAD and stress responses. It is therefore of great interest to know whether Ysy6p and RAMP4 are genuine homologs and to determine which specific processes Ysy6p is involved with.

1.7.2 The EMC complex

The ER Membrane Complex (EMC) is composed of six ER associated proteins (EMC1-6) and was first identified on the basis of induction level of the UPR in deletion strains for members of this complex (Jonikas et al., 2009). In this study the relative expression level of GFP under the control of the UPRE in a single deletion strain for members of this complex was compared to double deletion strains for members of the complex. It was shown that the double mutants had consistently lower level of UPR induction than expected thereby demonstrating that these genes formed a positive epistatic cluster. The members of the EMC complex were coimmunoprecipitated as a stoichiometric complex. It was noted by the authors that the genetic pattern of deletion of members of the EMC complex most resembled that of a strain expressing the constitutively misfolded transmembrane protein Sec62-1 and lead to the hypothesis that the EMC complex has a function in protein folding. Recently, it has been reported that in mammalian cells that homologs of the EMC (mEMC) were coimmunopreciptated as complexes from both digitonin and Triton X-100 solubilised lysates (Christianson et al., 2012). Interestingly the mEMC complex showed physical interactions with proteins which form promiscuous physical interactions with proteins associated with ERAD. These include Derlin-1, Derlin-2, which are homologs of Der1p in yeast, and UBAC2, homolog of Dsk2p. The mEMC is therefore associated with ERAD. It was also observed that the mEMC members are upregulated in the presence of tunicamycin and therefore can be considered UPR targets. In yeast only *EMC4* has been shown to be upregulated by the UPR (Travers et al., 2000). However, this does not mean that other members of the yeast are not UPR targets as in this latter study due to the stringency of criteria used the well characterised UPR target Kar2p is not annotated as being a UPR target (Cox et al., 1993). Interestingly, a genetic interaction between *HAC1* and *EMC1*, *EMC3*, *EMC4* and *EMC5* when cells are stressed with tunicamycin but not in the presence of Dithiotheritol (DTT) which also causes induction of the UPR by oxidative stress (Bircham et al., 2011, Travers et al., 2000). This study also found that deletion of EMC complex members lead to defective forward trafficking of the integral plasma membrane protein Mrh1p-GFP with marked perinuclear staining.

In addition to the interactions previously discussed the EMC complex has multiple other genetic and physical interactions with processes concerning the secretory pathway. All members of the *EMC* complex display genetic interactions with Kar2p,

Ssh1p and the GET complex (Hoppins et al., 2011, Costanzo et al., 2010, Vembar et al., 2010, Jonikas et al., 2009, Schuldiner et al., 2005). Lastly, it has been shown that the EMC complex has a genetic interaction with *YSY6* via *EMC5* (Schuldiner et al., 2005, Ito et al., 2001). There is therefore strong genetic evidence that the EMC complex function concerns protein biogenesis.

1.8. PROJECT AIM

As has been previously discussed there is evidence that RAMP4 is involved in protein biogenesis. In addition, the putative yeast homolog of RAMP4, Ysy6p, has been demonstrated to be involved in similar processes. However, the evidence surrounding this is either indirect as is the case for studies of genetic interaction or is from a study involving xenobiotic expression of Ysy6p in *E. coli* (Costanzo et al., 2010, Jonikas et al., 2009, Yu et al., 2008, Sakaguchi et al., 1991). This therefore raises the tantalising question of whether Ysy6p is indeed a true homolog of RAMP4. For example, is Ysy6p also tightly associated with ribosomes and can it also be implicated in degradative or folding processes? In addition, Ysy6p has been shown to have a genetic interaction with the recently identified EMC complex, the function of which remains largely unknown. However, studies to date suggest involvement of the EMC complex in similar processes as the mammalian homolog of Ysy6p, RAMP4, such as stress responses, protein folding and ERAD (Christianson et al., 2012, Bircham et al., 2011, Jonikas et al., 2009). Studying the function of the EMC complex in parallel to Ysy6p may therefore give reciprocal insights into the function of these largely uncharacterised proteins.

CHAPTER 2: Materials and Methods

All Chemicals and reagents unless stated were obtained at analytical grade from Aldrich, Becton Dickinson and Company (BD), Calbiochem, Roche, Sigma, Sigma-Aldrich, Melford and New England Biolabs.

2.1 Strains and Growth conditions

2.1.1. *Escherichia coli* strains

Two different *Escherichia coli* strains were used in this study as described in table 2.1.

Table 2.1 *Escherichia coli* strains used in this study

Strain	Genotype	Source/ reference
DH5α	*F⁻ϕ80dlacZΔM15 Δ(lacZYA-argF) U169 deoR recA1 end A1 hsdR17 (r_k, m_k^+) sup E44 λ⁻ gyrA96 relA1*	(Hanahan, 1983) (Hanahan, 1983)
XL10-Gold	Tetr Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB lacI*qZΔ*M15* Tn*10* (Tetr) Amy Camr]	Stratagene

2.1.2 Media and growth conditions

E. coli cells were grown in liquid culture at 37°C, 200 rpm in Luria-Bertani (LB) media (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride) or at 37°C on LB agar (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) sodium chloride, 2% (w/v) Bacto™ agar) and supplemented with 100 mg/L ampicillin where appropriate.

2.1.3. *Saccharomyces cerevisiae* strains

Saccharomyces cerevisiae strains used in this study are listed in table 2.2

Table 2.2 *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Source/ reference
BW792	*MATa his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	Dr. Barrie Wilkinson
Δ*emc5*Δ*ysy6 (a)*	*emc5::KanMX4 ysy6::KanMX4*	
BW793 Δ*emc5*Δ*ysy6 (b)*	*MATalpha his3Δ1 leu2Δ0 ura3Δ0 emc5::KanMX4 ysy6::KanMX4*	Dr. Barrie Wilkinson
BY4741	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	(Winzeler et al., 1999)
BY4741 Δcue1	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 cue1::KanMX4*	(Winzeler *et al.,* 1999)
BY4741 Δemc1	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 emc1::KanMX4*	(Winzeler *et al.,* 1999)
BY4741 Δemc2	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 emc2::KanMX4*	(Winzeler *et al.,* 1999)
BY4741 Δemc3	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 emc3::KanMX4*	(Winzeler *et al.,* 1999)
BY4741 Δemc4	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 emc4::KanMX4*	(Winzeler *et al.,* 1999)
BY4741 Δemc6	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 emc6::KanMX4*	(Winzeler *et al.,* 1999)
BY4741 Δder1	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 der1::KanMX4*	(Winzeler *et al.,* 1999)
BY4741 Δhrd1	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 hrd1::KanMX4*	(Winzeler *et al.,* 1999)
BY4741 Δlhs1	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 lhs1::KanMX4*	(Winzeler *et al.,* 1999)
BY4741 Δsec71	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 sec71::KanMX4*	(Winzeler *et al.,* 1999)
BY4741 Δ*ubc7*	*MATa his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 ubc7::KanMX4*	(Winzeler *et al.,* 1999)
BY4742	*MATalpha his3Δ1 leu2Δ0 lys2Δ ura3Δ0*	(Winzeler *et al.,* 1999)
BY4742 Δemc5	*MATalpha his3Δ1 leu2Δ0 lys2Δ ura3Δ0 kre27::KanMX4*	(Winzeler *et al.,* 1999)
BY4742 Δysy6	*MATalpha his3Δ1 leu2Δ0 lys2Δ ura3Δ0 ysy6::KanMX4*	(Winzeler *et al.,* 1999)
CST211	*MATa prc1-407, prb1-1122, pep4-3, leu2, trp1, ura3*	Prof. Colin Stirling
RPL25-GFP	*MATalpha ade2-1 his3-11, 15 ura3-52 leu2-3, 112 trp1-1 can1-100rpl25::HIS3* [*pRPL25GFP-TRP*]	(Hurt et al., 1999)
RS453	*MATalpha ade2-1 his3-11, 15, leu2-3, 122, trp1-1 ura3-52*	(Segref et al., 1997)
Sec62-1	*MATa* leu2 ura3 his4 sec62-1	Jane Dalley

2.1.4. Media and growth conditions

S. cerevisiae strains were grown at 30°C, 200 rpm in either rich YPD media (1% (w/v) Gibco BRL™ select yeast extract, 2% (w/v) Bacto™ peptone 2% (w/v) glucose) or minimal SD media (0.675% (w/v) Difco™ Yeast Nitrogen Base, 2% (w/v) glucose) supplemented with 0.002% (w/v) adenine, 0.004% (w/v) methionine, 0.003% (w/v) lysine, 0.002% (w/v) tryptophan, 0.01% (w/v) leucine, 0.002% (w/v) uracil and 0.002% (w/v) histidine.

2.2 Plasmids

Several plasmids were used in this study described in table 2.3.

Table 2.3 Plasmids used in this study

Plasmid	Genotype	Source/ reference
pAS2	*HMG1Δ7-SUC2-URA3* fusion in pRS315	A. Selkirk
pR16	SalI,SpeI 2022bp fragment containing SSH1-myc ligated into pRS316	Prof. C. Stirling
pRH244	N-terminus 6myc tagged HMG2 under TDH3 promoter containing Amp and URA selection.	(Hampton et al., 1996)
pMP234	PHO8-URA3 fusion in pRS315	(Dalley *et al.,* 2008)
pMR12	CPY-URA3 fusion in Yep351	Dalley *et al.,* 2008
pRS315	Yeast shuttle vector –ori-bla CEN6-ARSH4 containing Amp and *LEU2* markers	(Sikorski and Hieter, 1989)
pRS315-Ysy6N	pRS315 containing Ysy6 with C terminal opsin tag under the control of the MET25 promoter	Prof. B. Schwappach
pRS315-Ysy6NQ	pRS315 containing Ysy6 with C terminal glycosylatable opsin tag under the control of the MET25 promoter	Prof. B. Schwappach
pRS315-Ysy6NQT1	pRS315 containing Ysy6 N terminal truncation 1 with C terminal glycosylatable opsin tag under the control of the MET25 promoter	This study
pRS315-Ysy6NQT2	pRS315 containing Ysy6 N terminal truncation 2 with C terminal glycosylatable opsin tag under the control of the MET25 promoter	This study
pRS315-Ysy6NQT3	pRS315 containing Ysy6 N terminal truncation 3 with C terminal glycosylatable opsin tag under the control of the MET25 promoter	This study
pRS416	Yeast shuttle vector –ori-bla CEN6-ARSH4 containing Amp and *URA3* markers	(Sikorski and Hieter, 1989)
pRS416-YOR1-GFP	MET25-*YOR1-GFP*, Amp and URA markers	Prof. B. Schwappach

2. 3 Oligonucleotides

Oligonucleotide primers used in this study are listed in table 2.4

Table 2.4 Primers used in this study

Screening primers	
Emc1Screening_F	TCAACACTTTCATCCTCCCCT
Emc1Screening_R	GCCAAAATAAGGAGCCTTGA
Emc2screening_F	TTCGTATCGCGTGATGTGTT
Emc2screening_R	CCCGTAGCAGGATCAAATCAT
Emc3screening_F	ACGAACCGAATTACCTCACCT
Emc3screening_R	TCCAAATACTGAATGCGTCG
Emc4screening_F	GAAGACGCAAAGGGCAATAA
Emc4screening_R	TCAAGTTGTGAAGCTGGTCAA
Emc5screening_F	CAAGCCCATTCAATCGTCAT
Emc5screeningR	ATGAAAGGCGTCTGTGTTGGT
Emc6screening_F	GTCTTTGTGAGAAAGGTGGGT
Emc6Screening_R	CTGCAAAAATCTTTGCGCTC
Sequencing primers	
Ysy6internal_F	GCTAATGCCAAGTTTAACAAGA
Ysy6internal_R	CCACCTACGAGAAGAAACAGAA
Primers for modification of plasmid pRS315-Ysy6NQ	
Ysy6NQT1_F	AGACAAAGACTGGCTAATGC
Ysy6NQT2_F	CTGGCTAATGCTAAGTTTAACAAG
Ysy6NQT3_F	AACAAGAATAACGAAAAGTATAG
Ysy6NQ_R	(Phos)GGCCATGGTGGATCCAC

2.4 DNA manipulations and extraction

2.4.1 Plasmid extraction from *E. coli*

A single colony was propagated overnight in liquid LB media containing ampicillin and purified using the QIAGEN® mini or midi purification kit according to the manufacturer's instructions. Plasmid purity and concentration were then characterised by agarose gel electrophoresis as described in 2.4.2 and spectrophotometry at a wavelength of 260 nm.

2.4.2 Agarose DNA Gel electrophoresis

DNA was run on 1% (w/v) agarose gels prepared with TAE buffer (48.4 g/l Tris-HCl, 1.3% (v/v) acetic acid, 2% (v/v) ethidium bromide, 100mM EDTA at pH 8.0) 6x DNA loading buffer (0.42% (w/v) bromophenol blue, 25% (w/v) ficoll, 10 mM EDTA) was added to each sample. Gels were run at 60 V and visualised in Uvitec UV transilluminator.

2.4.3 Sequencing of plasmid DNA

Sequencing was carried out by the University of Manchester sequencing facility. Plasmid DNA was sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit and the Applied Biosystems™ 3730 DNA Analyzer using 400 ng of plasmid template and 4 pmols of primer.

2.4.4 Genomic DNA extraction

Cells were propagated in 5 ml YPD media overnight as described in 2.1.4. The cells were collected by centrifugation at 6000 x g for 1 minute, washed with dH_20 and resuspended in 400 µl Buffer A (2% (v/v) Triton X-100, 1% (w/v) SDS, 0.1 M Nacl, 1 mM EDTA, 10 mM Tris-HCl pH 7.6). To the sample 400 µl of 0.5 mm glass beads (Biospec Products Inc.) and 400 µl of phenol:chloroform:isoamyl (25:24:1) were then added. The sample was then vortexed for 3 minutes and 200 µl TE (10mM Tris-HCl, 1 mM EDTA pH 7.5) was then added. The sample was then centrifuged at 16 000 x g for 10 minutes. Following centrifugation, 500 µl of the top layer was then removed and chloroform extracted. Chloroform extraction was carried out by adding 400 µl of chloroform to the sample followed by centrifugation at 16000 x g for 10 minutes. The top layer was then removed and subjected to a further two rounds of cholorform extraction. DNA was precipitated by addition of 1 ml of 100% (v/v) ethanol to the sample followed by vortexing and centrifugation at 16000 x g for 10 minutes. The pellet was then washed with 70% (v/v) ethanol and resuspended in 50 µl TE. RNA was then digested by addition of 0.5 µl of 10 mg/ml RNase A and incubated for 1 hour at 37°C.

2.4.5. PCR of genomic DNA

Genomic DNA was extracted as described in 2.4.4 and reactions were carried out using 5µg genomic DNA, 2 U Taq DNA polymerase (Roche), 500 µM of each dNTP, 1x taq PCR buffer, 2 µM forward primer, 2 µM reverse primer. PCR was carried out with a 2 minute denaturation at 98°C followed by 30 cycles which included 98°C for 30 seconds, 50°C for 45 seconds and 72°C for a time dependent on the fragment size (2 minutes for products under 2 Kbp and 3 minutes for products over 2Kbp). Cycling was followed by a final extension of 7 minutes at 72°C.

2.4.6. Truncation of Ysy6NQ

A phosphorylated reverse primer and three forward primers, described in table 2.3 were designed for PCR amplification of the pRS315-Ysy6NQ fragment. Reactions were carried out using 20ng plasmid DNA, 1x Phusion® HF buffer, 200 µM of each dNTP, 1U of Phusion® DNA polymerase in a final reaction volume of 50µl. Hot start PCR was carried out with 4 minutes at 98°C of denaturation prior to addition of DNA polymerase. This was followed by denaturation for a further 2 minutes at 98°C and 30 cycles which included 98°C for 30 seconds, 30 seconds at variable temperature depending on the forward primer (Ysy6NQT1_F: 61°C, Ysy6NQT2_F: 55°C, Ysy6NQT3_F: 53°C) and 72°C for 6 minutes. Cycling was followed by a final extension of 10 minutes at 72°C.

Samples were then digested with Dpn1 for 2 hours at 37°C and run on a 0.6% (w/v) agarose gel and the band of expected Mw was gel extracted using the QIAquick gel extraction kit. Blunt end ligation was then carried out using T4 DNA ligase. *XL10-Gold E. Coli* cells were then transformed and plated onto selective media. Transformants were propagated and plasmid DNA extracted. Plasmid identity was verified by digestion with EcoRV for 1 hour followed by sequencing using internal primers to *YSY6* as described in 2.4.3.

2.5 Preparation of DH5α electro-competent cells

DH5α *Escherichia coli* cells were propagated overnight in 1 ml LB media overnight at 37°C and resuspended to an OD_{600} of 0.1 and propagated to an OD_{600} of 0.4. Cells were

then cooled on ice for 30 minutes followed by collection by centrifugation at 4°C for 5 minutes at 2300 x g. Cells were then resuspended in ice cold dH_20 followed by collection of cells by centrifugation at 4°C for 5 minutes at 2300 x g and the process repeated twice. The cells were then resuspended in 2 ml ice cold 10% (v/v) glycerol followed by collection of cells by centrifugation at 4°C for 5 minutes at 2300 x g. The cells were then resuspended in 300 µl of ice cold 10% (v/v) glycerol and frozen with liquid nitrogen and stored at -80°C.

2.6. Transformation of *Escherichia coli*.

Escherichia coli XL10-Gold® ultracompetent cells (Stratagene) were transformed as described by the manufacturer. DH5α *Escherichia coli* were transformed by electroporation. A 40 µl aliquot of cells was defrosted on ice. 0.5 µg of plasmid DNA was then added to the cells and incubated on ice for 5 minutes. Cells were then placed in a pre-chilled 0.2 cm gap Bio-Rad Gene Pulser® electroporation cuvette. Cells were then electroporated at 2.1 Kv and 200 Ω. Electroporated material was then incubated in 500 µl LB and incubated for one hour at 37°C and 200 rpm. Cells were then collected by centrifugation at 2300 x g and resuspended in 100 µl LB. Cells were then plated on LB agar plates containing 100 µg/ml ampicillin and incubated overnight at 37°C.

2.7 Transformation of *Saccharomyces cerevisiae*

A toothpick head of *S. cerevisiae* cells freshly grown on YPD agar were washed with 1 ml sterile dH_20, resuspended in 10 µl of 2 µg/ml herring testes DNA and 1-2 µg plasmid DNA. This was followed by addition of 600 µl of 40% (w/v) PEG 3350, 100 mM LiAc pH 7.5, 10 mM Tris-HCl, 1 mM EDTA pH 7.5 and the reaction mixture left for 6 to 18 hours at room temperature. The cells were then harvested and resuspended in 100 µl 10 mM Tris-HCl, 1 mM EDTA pH 7.5. The cells were then plated on appropriate selective media and incubated at 30°C. Transformants were then purified twice by streaking a single colony onto appropriate selective SD agar and incubated at 30°C. As a negative control the above stated method was also carried out without the addition of plasmid DNA.

2.8 Protein Biochemistry

2.8.1 Antibodies

Several antibodies were used in this study as described in table 2.5.

Table 2.5 Antibodies used in this study

Antibody	Animal raised in	concentration	Reference/ source
GFP	Rabbit	1:3000	D. Görlich
Kar2p	Sheep	1:5000	(Frey et al., 2001)
Myc	Mouse	1:5000	Sigma
Opsin	Mouse	1:1000	S. High
Pho8p	Rabbit	1:100	Invitrogen
Rps3p	Rabbit	1:50 000	M. Pool
Sec61p	Rabbit	1:5000	R. Scheckman
Ysy6p	Rabbit	1:500	B. Schwappach
Zwf1p	Rabbit	1:5000	Sigma
HRP-Anti mouse Ig	Goat	1:5000	Sigma
HRP-Anti Rabbit Ig	Goat	1:5000	Sigma
HRP-Anti Sheep Ig	Donkey	1:5000	Sigma

2.8.2 SDS-PAGE and Western blotting

Prior to SDS-PAGE samples were resuspended in sample buffer (187.5 mM Tris HCl pH 6.8, 6% (w/v) SDS, 30% (w/v) glycerol, 0.03% (w/v) bromophenol blue, 2% (v/v) β-mercaptoethanol) with proteinase inhibitors (chymostatin (5 μg/ml), leupeptin (10 μg/ml), aprotinin (10 μg/ml) and pepstatin (1.4 μg/ml); or Complete EDTAfree Protease inhibitor cocktail (Roche). SDS-PAGE was carried out according to standard protocols (Laemmli, 1970). Gels were composed of an 'upper' stacking gel and a 'lower' resolving gel. The stacking gel contained 4% (v/v) Acrylamide:biscaryl (Protogel®, national diagnostics), 0.125 mM Tris pH 6.8, 0.1% (w/v) SDS. The resolving gel contained the stated percentage of Acrylamide:biscaryl and 0.4 M Tris pH 8.8, 0.1% (w/v) SDS. Gels were run in running buffer (0.2 M glycine, 25 mM Tris, 0.1 % (w/v) SDS) using a Biorad Mini-PROTEAN®3 cell at 38 mA for 45 minutes. Proteins were transferred onto Whatman® Protran® 0.4 μm nitrocellulose membrane using a Biorad Trans-Blot® semi-dry transfer cell. Buffers used for the transfer: anode buffer 1 (30mM Tris-base, 20% (v/v) methanol) anode buffer II (300mM Tris-base, 20% (v/v) methanol) and cathode buffer (25mM Tris-base, 40mM 6-aminohexanoic acid, 20% (v/v) methanol, 0.01% (w/v) SDS). Blotting was carried out at 15 V for 1 hour. Nitrocellulose membranes were then washed with dH_20 and stained with Ponceau S solution (0.2% (w/v) Ponceau S, 5% (v/v) acetic acid). Visual inspection of Ponceau

S staining was used as a control for total protein loaded in each lane. Ponceau S was then washed off with dH_20 and PBSN (0.14 M NaCl, 10 mM NaH_2PO_4, NP-40 alternative pH 7.2). The membrane was then blocked with 10% (w/v) milk (Marvel™, dried skimmed milk) PBSN for 1 hour followed by incubation in 2% (w/v) milk PBSN containing the appropriate primary antibody. The membrane was then washed five times for five minutes with PBSN followed by incubation with PBSN containing 2% (w/v) milk and the appropriate IgG-peroxidase (Sigma) for 1 hour. The membrane was then washed again five times for five minutes with PBSN. Immobilon™ western chemiluminescent HRP substrate (Millipore) was then applied to the membrane and visualised either by exposing to Kodak BioMax MR, Kodak BioMax XAR scientific imaging film.

2.8.3. Methanol precipitation of proteins

Methanol precipitation was carried out as described by (Wessel and Flugge, 1984). In brief, 0.4 ml of methanol was added to 0.1 ml of sample. The sample was then vortexed for 10 seconds and centrifuged at 9000 x g for 10 seconds followed by addition 0.2 ml of chloroform. The sample was then vortexed and 0.3 ml of dH_20 added followed by vortexing and centrifugation at 9000 x g for 1 minute. The upper phase was discarded and 0.3 ml of Methanol added. The sample was then vortexed, centrifuged for 2 minutes at 9000 x g and the supernatant removed. The sample was then air dried at 22°C and resuspended in sample buffer and analysed by SDS-PAGE as described in 2.8.2.

2.8.4. Trichloroacetic acid precipitation of proteins

Trichloroacetic acid (TCA) was added to a concentration of 10% (v/v) and left on ice for 10 minutes. The cells were then pelleted by centrifugation at 8800 x *g* for 2 minutes and resuspended in 1.5 ml acetone. The cells were then pelleted again by centrifugation at 8800 x g and the acetone removed. The pellet was then left to air dry at 24°C and then resuspended in sample buffer and analysed by SDS-PAGE and western blotting as described in 2.8.2.

2.8.5. Protein extraction by NaOH lysis

Strains were grown in appropriate liquid media. Cells were then harvested by centrifugation and washed with dH_2O. Cells were then lysed with 200 µl 0.1 M NaOH, 2% (v/v) β-mercaptoethanol and Trichloroacetic acid precipitated as described in 2.8.4.

2.8.6. Cycloheximide chase assay

Strains were transformed as described in 2.7 with plasmid pRH244 encoding for the Hmg2-6myc construct. The strains were then propagated in selective media to stationary phase then resuspended in rich YP media to an OD_{600} of 0.1 and grown to an OD_{600} of 0.4-0.6. Cycloheximide was then added to a final concentration of 0.5mg/l. The OD_{600} was then measured and 3 OD_{600} units of cell culture was then taken 2 minutes after addition of cycloheximide and placed in a prechilled falcon tube and Sodium azide added to a final concentration of 10 mM. This constituted the T=0 time point. Subsequent aliquots were taken periodically, as stated, for three hours. The samples were then lysed by sodium hyrdroxide lysis and TCA precipitation as described in 2.8.4 and analysed by SDS-PAGE and western blotting as described in 2.8.2 using the anti-myc and anti-zwf1p antibodies.

2.8.7. Preparation of the P16 membrane enriched cell extract.

A single colony was picked and propagated to a high optical density in 5-10 ml of liquid YPD or selective SD media. The culture was then resuspended to an OD_{600} of 0.1 or 0.2 and grown to an OD_{600} of 0.5-0.6 in YPD. Cycloheximide was then added to the culture to a final concentration of 100 mg/l and incubated for a further 15 minutes. Cells were then harvested, washed with dH_20 containing cycloheximide (100mg/L) and resuspended in Low Salt (LS) buffer (20 mM Hepes-KOH pH 7.2, 100 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 2 mM DTT, 100 µg/ml cycloheximide, 5 µg/ml chymostatin, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1.4 µg/ml pepstatin) in a 1/100 LS buffer to culture volume ratio. Two cell pellet volumes of 0.5mm glass beads (Biospec Products Inc.) were then added to the sample and the cells were lysed by vortexing for 3 minutes at 4°C. The lysate was then separated from the beads by suction. The lysate was then centrifuged at 1200 x *g*. The supernatant was

then centrifuged again at 16000 x g for 20 minutes at 4°C. The sedimented material was then resuspended in LS buffer without Dithiothreitol and centrifuged for 20 minutes at 16000 x g. The sedimented material was then resuspended in LS buffer without Dithiothreitol.

2.8.8. Chemical crosslinking

Crosslinking was carried out by adding disuccinimiyl suberate (DSS) or m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) obtained from Thermo Fisher Scientific. Crosslinking with MBS and DSS was carried out using the P16 membrane extract prepared as described in 2.8.7. Crosslinking was performed using the P16 membrane preparation at a final concentration of 0.7 OD_{600}/µl of cell culture equivalent unless otherwise stated. The P16 membrane preparation was treated with MBS or DSS to the stated final concentration or mock treated with DMSO to a maximum concentration of 3.6% (v/v). This was followed by incubation at 24°C for 20 minutes unless otherwise stated. Crosslinking with MBS was quenched by addition of 0.1M β-mercaptoethanol, 0.1M glycine to a final concentration of 12.5 mM β-mercaptoethanol, 12.5 mM glycine unless stated otherwise. Crosslinking with DSS was quenched by addition of 0.1M glycine to a final concentration of 12.5 mM. The sample was then resuspended in an appropriate amount of 3 x sample buffer and analysed by SDS-PAGE followed by western blotting as described in 2.8.2 unless otherwise stated.

2.8.9. Denaturing immunoprecipitation

The P16 membrane preparation prepared as described in 2.7.8 was pelleted and resuspended in 100 µl TSE buffer (25 mM Tris-Hcl pH8, 0,1% (w/v) SDS and 25 mM EDTA) and denatured for 15 minutes at 65°C. Samples were the briefly cooled on ice prior to addition of 1 ml ice cold IP buffer A (10mM Tris-Hcl pH 8.0, 0.3% (v/v) Triton X-100, 140 mM Nacl, 1 mM EDTA, Mini EDTA free proteinase inhibitors (Roche)) The sample was then vortexed and centrifuged at 16 000 x g for 5 minutes at 4°C. The supernatant was then removed to a new eppendorf tube.

Binding of the antibody to the antigen was carried out using two methods as stated. In the first method, an appropriate amount of antibody was added to the supernatant as

stated. The sample was then mixed by gentle rotation for 16 hours at 6°C. The sample was then centrifuged at 16000 x g for 5 minutes at 4°C. The supernatant was then removed to a new eppendorf with an appropriate amount of protein A sepharose. The sample was then mixed by gentle rotation for 1 hour at 6°C. In the second method, stated amounts of antibody and protein A sepharose were bound to each other in 1 ml IP buffer A at 6°C for one hour and rotational mixing. The sample was then centrifuged for 1 minute at 16 000 x g and the supernatant discarded. The supernatant containing the P16 membrane preparation was then added to the Ysy6p antibody bound to protein A sepharose. The sample was then mixed by gentle rotation of 16 hours at 6°C.

Following binding of the antigen to the antibody the sample was centrifuged for 1 minute at 16 000 x g and the supernatant removed. The beads were then washed twice with 1 ml IP buffer A, twice with buffer B (10 mM Tris-HCl pH 8.0, 0.4% (v/v) NP-40, 500 mM Nacl, 1 mM EDTA) and once with 10 mM Tris-HCl pH 8.0. The protein was then eluted by adding 15 µl 3x SDS-PAGE sample buffer followed by incubation at 65°C for 10 minutes. Samples were then analysed by SDS-PAGE followed by western blotting and where stated by silver staining and mass spectrometry. SDS-PAGE and western blotting were performed as described in 2.8.2.

Silver staining was performed using the Bio-Rad Silver Stain Plus Kit according to manufacturer's instructions. In-gel digestion of silver stained protein band and masspectrometry was carried out by the University of Manchester protein identification service. In gel digestion was performed by incubating the excised band for 5 minutes in acetonitrile. The acetonitrile was removed by centrifugation at 1500 rpm of the excised band in a perforated 96 well plate followed by drying in a vacuum centrifuge. The sample was then incubated in Solution 1 (10 mM DTT, 25 mM NH_4HCO_3) for 1 hour at 56 °C. Solution 1 was then removed by centrifugation for 1 minute at 1500 rpm and incubated in solution 2 (55 mM Idoacetamide, 25 mM ammonium bicarbonate) for 45 minutes at room temperature in the dark. Solution 2 was then removed by centrifugation for 1 minute at 1500 rpm and the gel pieces incubated with 25 mM NH_4CO_3 for 10 minutes. The sample was then centrifuged for 1 minute at 1500 rpm followed by incubation for 5 minutes in acetonitrile. The sample was then centrifuged for 1 minute at 1500 rpm followed by incubation for 5 minutes in NH_4CO_3 followed by centrifugation for 1 minute at 1500 rpm and incubation in acetonitrile for 5 minutes.

The sample was then dried by centrifugation for 1 minute at 1500 rpm followed by centrifugation in a vacuum centrifuge. The sample was then digested with trypsin (Promega) (1.25 mg/L trypsin, 25 mM NH_4CO_3) for 45 minutes in a water bath at 0°C. This was followed by overnight incubation at 37°C. The peptides were then extracted by incubation in 20 mM NH_4CO_3 for 20 minutes followed by centrifugation for 1 minute at 1500 rpm. The extraction was then repeated twice with Solution 3 (5% (v/v) formic acid, 50% (v/v) acetonitrile). The extracts were then pooled, dried and resuspended in 5% (v/v) acetonitrile, 0.1% (v/v) formic acid. The sample was then analysed by LC-ESI-MS/MS. Data produced was searched using Mascot (Matrix Science UK), against the SWISSPROT database and the YEAST orf database and validated using Scaffold (Proteome Software, Portland, OR).

2.8.10. Ribosome sedimentation

For ribosome sedimentation buffers and solutions were made using dH_20 treated with 0.02% (v/v) diethylpyrocarbonate for 18 hours at 30°C followed by autoclaving at 121°C for 45 minutes.

A single colony was picked and propagated to a high optical density in 5-10 ml liquid YPD or selective SD media as stated in 2.1.4. The culture was then resuspended to an OD_{600} of 0.1 and grown to an OD_{600} of 0.5-0.6 in YPD. Cycloheximide was then added to the culture to a final concentration of 100 mg/l and incubated on ice for 20 minutes. Cells were then harvested, washed with dH_20 containing cycloheximide (100 mg/L) and resuspended in High Salt (HS) buffer (20 mM Hepes-KOH pH7.6, 500mM potassium acetate, 5mM magnesium acetate, 1 mM EDTA, 2 mM 2-mercaptoethanol, 100 µg/ml cyclohexamide, 5 µg/ml chymostatin, 10 µg/ml leupeptin, 10 µg/ml aprotinin and 1.4 µg/ml pepstatin) in a ratio of 200 OD_{600} units/ml HS buffer.

An equal volume of 0.5 mm acid washed glass beads (Sigma) was added to the HS buffer and vortexed for 10 minutes at 4°C. The supernatant was centrifuged at 1 200 x g for 2 minutes. The supernatant, unless otherwise stated, was solubilised using 3% (w/v) CHAPS (Calbiochem) and 10% (v/v) Glycerol for 60 minutes on ice. The lysate was then centrifuged at 16 000 x g for 20 minutes. An aliquot of the supernatant was kept and the rest layered on top of a 400 µl sucrose cushion (HS buffer, 1% (w/v) CHAPS and 15% (w/v) sucrose). The samples were centrifuged in a TLA 100.3 rotor at

70 000rpm (200 000 rcf) for one hour at 4°C using 1.5 ml polyallomer Microfuge® tubes. The pellet was re-suspended in HS buffer with 1% (w/v) CHAPS. Samples were methanol precipitated unless otherwise stated as described in 2.8.3 and analysed by SDS-PAGE and western blotting as described in 2.8.2.

2.8.11 Polysome analysis

For polysome analysis the sedimented material was obtained as described in 2.8.10 and was resuspended in 300 µl HS buffer with 3% (w/v) CHAPS, 10% (v/v) glycerol. The sample was then centrifuged at 16 000g at 4°C for 5 minutes. The supernatant was then removed to a fresh eppendorf tube and 250 µl loaded onto a HS 1% (w/v) CHAPS 11.5 ml continuous 15-50% sucrose gradient. Gradients were made using the stepwise method previously reported by Luthe (1983). Briefly, 50% (w/v), 42% (w/v), 33% (w/v), 24% (w/v) and 15% (w/v) sucrose HS 1% (w/v) CHAPS buffers were made and sequentially layered and frozen with liquid nitrogen in a 14x95 mm Polyallomer centrifuge tube (Beckman Coulter). The gradient was then left to thaw overnight at 4°C. The sample was then centrifuged at 40 000 rpm (202 000 x g) in a SW40Ti rotor for 3 hours at 2°C. The gradient was then fractionated into 0.5 ml aliquots from top to bottom. Samples were then methanol precipitated unless otherwise stated as described in 2.8.3 and analysed by SDS-PAGE and western blotting as described in 2.8.2.

2.8.12 Proteinase K protection assay

The Proteinase K protection assay was carried out on membrane preparations made as described in 2.7.8 except that cells were not treated with cycloheximide. Membrane preparations were resuspended to 0.33 OD_{600} /µl of cell culture equivalent in Low Salt buffer 2 (20 mM Hepes-KOH pH 7.6, 100 mM potassium acetate, 5 mM magnesium acetate, 1 mM EDTA, 2 mM DTT, 2mM puromycin, 2 mM GTP, 7 mM EDTA). An aliquot of 30µl of sample was then taken and Proteinase K added to the stated concentration with or without 0.5% (v/v) Triton X-100. Samples were then left on ice for 35 minutes. Digestion was stopped by addition of 20% (v/v) trichloroacetic acid and left on ice for a further 10 minutes. The cells were then centrifuged at 8800 x g for 2 minutes and resuspended in 1.5 ml acetone. The cells were the spun down again and the acetone removed. The pellet was then left to air dry at room temperature and then

resuspended in sample buffer and analysed by SDS-PAGE and western blotting as described in 2.8.2.

2.9 Cellular methods

2.9.1 Competitive growth assays

A single colony was picked and propagated in YPD liquid media as stated in 2.1.4. The cells were grown to OD_{600}>1. The cells were then resuspended to an OD_{600} of 0.1 and grown to an OD_{600} of 0.4-0.6. The cells were then resuspended to an OD_{600} of 0.1, and 5 fold dilutions made in dH_20 and 4 µl aliquots were then plated onto appropriate selective or YPD media unless otherwise stated.

2.9.2 Microscopy

Cells were propagated in Minimal SD media lacking uracil and methionine overnight to stationary phase followed by propagation to an OD_{600} of 0.4-0.6 as stated in 2.1.4. The cells were then concentrated 50 times by centrifugation at 1500 x g for 5 minutes. A 4 µl aliquot was then spotted onto a 1 mm thick glass slide and covered with a Thickness N°1 Borosillate cover glass (Merck BDH).

Microscopy was carried out using the Applied Precision, Inc. Deltavision® system using the Olympus 100X objective with numerical aperture of 1.40 and the CoolSNAP_HQ2 camera. Images were taken with dimensions of 512x512 pixels and 2x2 binning. Transmitted light field images were taken with an exposure time of 0.05 seconds. Imaging of the Green fluorescent protein (GFP) signal was carried out using the Fluorescein isothiocyanate (FITC) filter with an exposure time of 0.025 seconds. Images were deconvolved using the SoftWoRx® image analysis software.

CHAPTER 3: Phenotypic characterisation of deletion strains of *YSY6* and members of the *EMC* complex

3.1 Introduction

High-throughput studies have highlighted genetic and physical interactions of *YSY6* and the *EMC* complex with factors associated with the UPR, ERAD, trafficking and the protein integration machinery (Bircham et al., 2011, Costanzo et al., 2010, Jonikas et al., 2009, Yu et al., 2008, Travers et al., 2000). Ysy6p was shown to be involved in protein integration by its ability to rescue defects in integration of the outer membrane protein OmpA in *E. coli* temperature sensitive mutants of SecY (Sakaguchi et al., 1991). The biochemical evidence of Ysy6p involvement is therefore indirect as it relies on data from xenobiotic expression of Ysy6p in prokaryotic cells. Much more evidence exists for involvement of the mammalian homolog, RAMP4, in secretory processes (Pool, 2009, Hori et al., 2006, Schroder et al., 1999, Yamaguchi et al., 1999, Görlich and Rapoport, 1993). It has been shown that RAMP4 is tightly associated with the Sec61p complex and membrane bound ribosomes (Yamaguchi et al., 1999, Görlich and Rapoport, 1993). Furthermore, RAMP4 has been shown to be recruited to the Sec61p complex during co-translational translocation of nascent proteins and also crosslinks to nascent proteins (Pool, 2009, Schroder et al., 1999). This therefore shows that the function of RAMP4 is tightly associated with protein integration at the ER. Deletion of murine *RAMP4*, similarly to deletion of *YSY6*, has been shown to induce the UPR (Jonikas et al., 2009, Yamaguchi et al., 1999). This suggests that deletion of *YSY6* and *RAMP4* causes the exposure of proteins to the UPR machinery. Interestingly, it has been reported that overexpression of RAMP4 in mammalian cells has a protective effect on proteins by inhibiting their degradation when the UPR is induced (Yamaguchi et al., 1999). This therefore implicates RAMP4 in degradative processes such as ERAD.

The yeast EMC complex was identified as a stoichiometric complex which has been proposed to be involved in protein folding (Jonikas et al., 2009). The mammalian EMC (mEMC) complex has been shown to coimmunoprecipitate with factors associated with the ERAD machinery and therefore forms part of the mammalian ERAD network (Christianson et al., 2012). Lastly, it has been shown that deletion of members of the

EMC complex leads to internal retention of the normally plasma membrane localised polytopic membrane protein Mrh1p-GFP in a manner which resembles ER localisation (Bircham et al., 2011, Huh et al., 2003). This therefore implicates the EMC complex in secretory processes.

Ysy6p and the EMC complex have therefore been implicated in multiple aspects of the secretory pathway. In order to further investigate the role that these genes play in the secretory pathway the impact of deleting these genes on secretory processes were investigated. The processes tested include protein integration and translocation by the co-translational and post-translational pathways, ERAD and forward trafficking by using relevant substrate proteins. In addition, phenotypic characterisation was carried out by assessing the relative fitness of the deletion strains with a focus on conditions which cause the induction of stress responses or report on the integrity of the cell wall or membrane. These conditions included growth on media containing SDS or elevated temperature (Leber et al., 2004, Trott and Morano, 2003, Bickle et al., 1998). These assays were also used to test whether any aggravating or alleviating effect could be observed for the double deletion of *YSY6* and *EMC5* and therefore rationalise the previously reported genetic interactions between these two genes (Schuldiner et al., 2005, Leber et al., 2004, Ito et al., 2001).

3.2 Investigating relative fitness of deletion strains of the *EMC* complex and *YSY6*

In order to characterise deletion strains of the *EMC* complex and *YSY6* serial dilution assays were performed. Cells were propagated to mid log phase in rich liquid YPD media followed by resuspension to an OD_{600} of 0.5 and five-fold serial dilutions made. The dilutions were then spotted onto YPD agar plates. Strains investigated included deletion strains of the *EMC* complex and *YSY6* deletion strains. In addition *YSY6* and *EMC5* double deletion strains were also investigated. As can be seen in figure 3.1, at 30°C the *WT* strains show growth at all dilutions with 4 to 20 colonies across *WT* strains at the highest dilution. The deletion strains gave rise to the same growth profile as WT strains at 30°C. In addition, no difference in colony morphology could be observed between the strains. This demonstrates that strains have a similar number colony forming units and therefore viability at 30°C.

It has previously been reported that deletion of *EMC5* results in decreased sensitivity to K1 killer toxin (Page et al., 2003). K1 Killer toxin acts by binding to the beta-glucan receptor of the yeast cell wall followed by channel formation in the cell membrane which leads to potassium efflux and cell death (Zhu and Bussey, 1991). The decreased sensitivity is therefore indicative of changes to the cell wall or membrane.

To further investigate whether the K1 killer toxin sensitivity was part of a wider cell wall phenotype, SDS sensitivity of the strains was assayed. Increased sensitivity to SDS has been demonstrated to relate to altered plasma membrane or cell wall biogenesis (Bickle et al., 1998). As shown in figure 3.1 deletion of the *EMC* complex members *EMC1*, *EMC2, EMC3* and *EMC6* showed a striking increase in sensitivity to 0.06% (w/v) SDS whereas deletion of *EMC5* gives rise to a moderate increase in SDS sensitivity. The *Δysy6* and Δ*emc4* strains did not show an increase in SDS sensitivity. Two double deletion strains for *EMC5* and *YSY6* were characterised (*BW792* and *BW793*). However, double deletion strains *BW792* (a) and *BW793* (b) have different sensitivity to SDS with strain *BW792* (a) being sensitive to 0.03% and 0.06% SDS whereas strain *BW793* (b) is insensitive to SDS.

In addition to SDS sensitivity, temperature sensitivity was investigated. As can be seen in figure 3.1 no defect in growth could be observed at 37°C or at 16°C. However, at the elevated temperature of 39.5°C strains Δ*emc1*, Δ*emc3*, Δ*emc6* displayed a temperature sensitive phenotype. None of the other strains showed any temperature sensitivity. Strains Δ*emc1*, Δ*emc3* and Δ*emc6* are therefore both temperature sensitive and SDS sensitive. However, strain *Δemc2* is only SDS sensitive but not temperature sensitive.

Due to the fact that the two *Δysy6Δemc5* strains showed variation in SDS sensitivity the strain identities were verified. As shown in appendix I the strains used were verified for deletion of *YSY6* by western blotting and no Ysy6p could be detected. The strains deleted for members of the *EMC* complex were also verified by PCR. This was carried out using upstream and downstream primers to the open reading frame. The amplified fragment sizes for the *WT* strain were then compared to the expected sizes for the deletion strains which contain the *KanMX* replacement cassette (Brachmann et al., 1998). All strains showed the expected shift in band sizes for correct insertion of the *KanMX* replacement cassette. This therefore confirmed the identity of the strains. In addition, this demonstrates that the discrepancy in SDS phenotypes of the *Δysy6Δemc5*

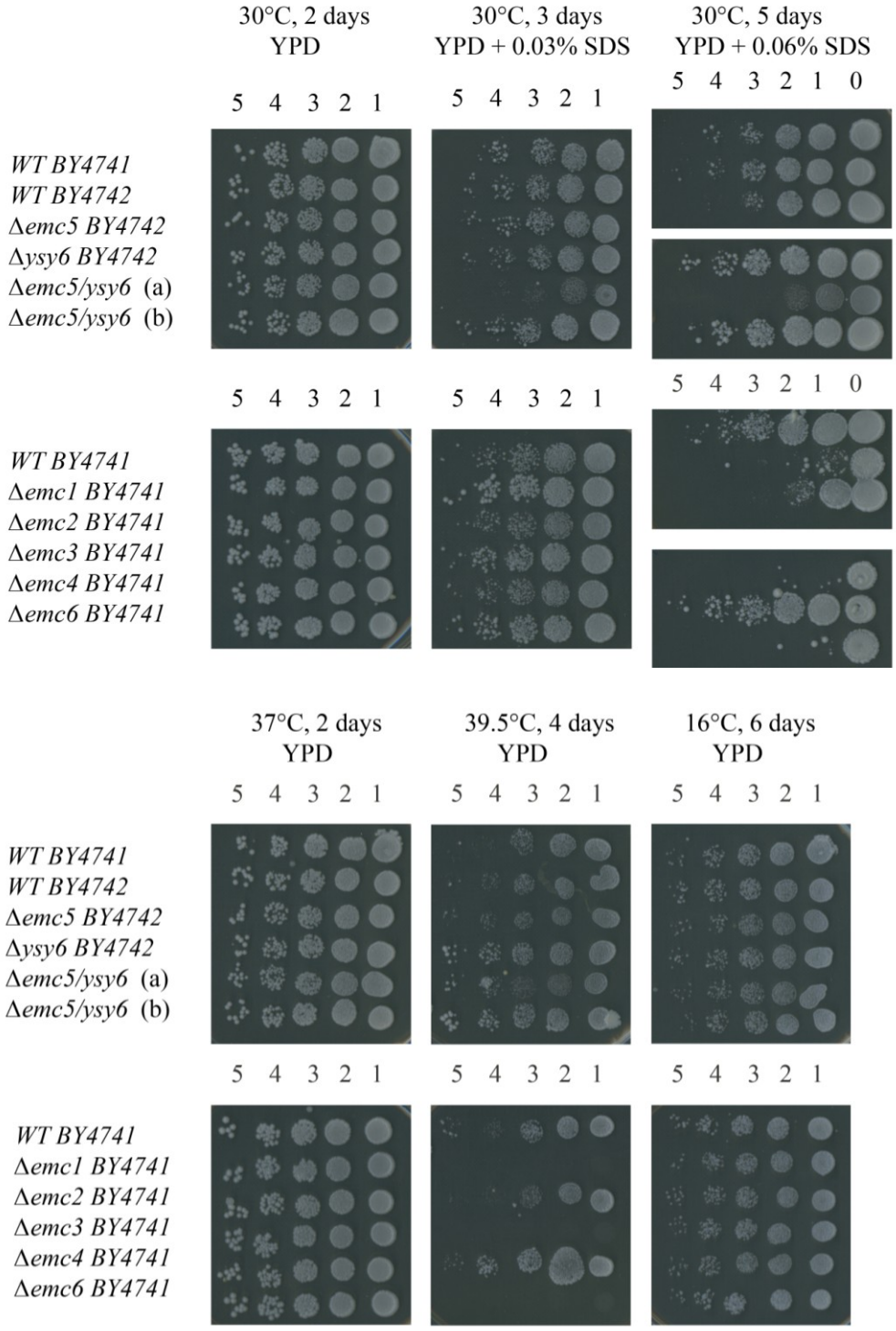


Figure 3.1 Phenotypic characterisation of deletion strains of *YSY6* and the *EMC* complex. Cells were propagated in rich YPD media to mid log phase and resuspended to an OD_{600} of 0.5. Five fold serial dilutions were then made (the number of dilutions is shown as numbers 0-5) and 5 µl aliquots were spotted onto YPD agar plates and 4 µl aliquots were spotted onto YPD plates containing 0.03% (w/v) or 0.06% (w/v) SDS. Plates made with YPD media alone were then incubated at 30°C, 39.5°C, 37°C and 16°C for the indicated number of days. Plates made with YPD media supplemented with SDS were incubated at 30°C only for the indicated number of days. Figures are representative of a minimum of two repeat experiments.

double deletion strains is not due to lack of deletion of the *YSY6* and *EMC5*. The exact causes of the discrepancy in SDS sensitivity between these two double deletion strains being difficult to assess both strains were retained for subsequent protein integration assays.

3.3 Investigation of protein integration at the ER in deletion strains of the *EMC* complex and *YSY6*

Studies in *E. coli* have demonstrated that xenobiotic expression of Ysy6p can rescue defects in the translocation of OmpA across the periplasm in a temperature sensitive mutant strain for SecY (Sakaguchi et al., 1991). Further evidence that Ysy6p may be involved in protein integration arises from studies of the mammalian homolog of Ysy6p, RAMP4. Yamaguchi et al., (1999) have shown that overexpresion of RAMP4 increases the glycosylation efficiency of the membrane proteins RAGE and CD8 and that RAMP4 can be crosslinked to the Sec61 complex. Similarly, Schroder et al., (1999) demonstrated that RAMP4 crosslinks to the MHC class II-associated invariant chain (Ii) during its integration into the ER. Ii is a type II co-translationally integrated membrane protein with an N-terminal transmembrane segment with C terminal glycosylation sites. RAMP4 was shown to crosslink Ii in a manner which was concomitant with the emergence of glycosylation sites into the ER lumen. However, it was shown that these glycosylation sites were not required for crosslinking to RAMP4. Instead, it was demonstrated that crosslinking depended on the presence of a hydrophobic sequence located directly upstream of the glycosylation sites. Pool (2009) also demonstrated that recruitment of RAMP4 to the Sec61p complex was dependent on the sensing of a transmembrane domain in the ribosomal exit tunnel. Lastly, Hori et al., (2006) demonstrated that proinsulin biosynthesis was delayed upon increase in glucose concentrations in RAMP4 knockout murine cells. This therefore suggests that RAMP4 is required efficient protein biogenesis at the ER.

In order to investigate whether Ysy6p plays a role in protein integration or translocation at the ER three protein substrates based on carboxypeptidase Y (CPY), Pho8p and Hmg1p were used. As shown in figure 3.2 the substrate proteins were fused to the *URA3* gene. The fusion made was such that if the protein was mis-integrated or mis-translocated across or into the ER the URA3 moiety would be exposed to the cytosol

and render the cells prototrophic for uracil. However, if protein biogenesis is unaffected then the URA3 moiety remains sequestered in the ER and cells remain auxotrophic for uracil (Dalley et al., 2008, Ng et al., 1996). The CPY-URA3 reporter was constructed by C terminally fusing *CPY* to *URA3* (Dalley et al., 2008). Pho8p is a type two membrane protein and consequently the *URA3* gene was fused to the N terminus of the Pho8p ORF (Dalley et al., 2008). The third reporter based on Hmg1p contains the first 463 residues of Hmg1p at its C terminus to the first 307 residues of invertase encoded by the *SUC2* gene (Stirling et al., 1992). This construct was then fused at its C terminus to the *URA3* ORF (Dalley et al., 2008). Only a fragment of the Hmg1p was used in the fusion construct which relates to a deletion in the seventh transmembrane domain. This was required in order to obtain the correct topology for a functional assay. The final construct is therefore HMG1Δ7-SUC2-URA3.

As has previously been discussed protein integration or translocation into or across the ER in yeast can occur via several pathways most notably the post-translational pathway and the co-translational pathway (Rapoport, 2007). The substrates were chosen to represent different classes of proteins. CPY is a soluble protein which is translocated into the ER by the post-tranlastional pathway and undergoes cleavage of its signal sequence (Ng et al., 1996, Valls et al., 1987). Pho8p is a type II single spanning membrane protein integrated by the co-translational pathway (Ng et al., 1996). Finally Hmg1p is a class I co-translationally integrated polytopic membrane protein (Stirling et al., 1992).

The protein integration assay was performed by transforming the reporter constructs into *YSY6* and *EMC* complex deletion strains. After transformation and purification of the transformants freshly grown cells were used to streak selective SD agar plates. Strains were streaked onto SD –Leu agar plates as a control for growth which selected for the plasmid marker. Strains were also streaked onto SD –Leu –Ura which selected for both the plasmid and exposure of the *URA3* moiety of the constructs to the cytosol. In addition to the query strains, isogenic *WT* negative control strains and positive control strains *sec62-1* and *rpl25-GFP* were transformed and streaked in parallel.

Mutations of *SEC62,* such as *sec62-1,* cause defects in protein translocation specific for proteins which use the SRP-independent post-translational pathway such as CPY-URA3 (Dalley et al., 2008, Ng et al., 1996). As can be seen in figure 3.3 all strains

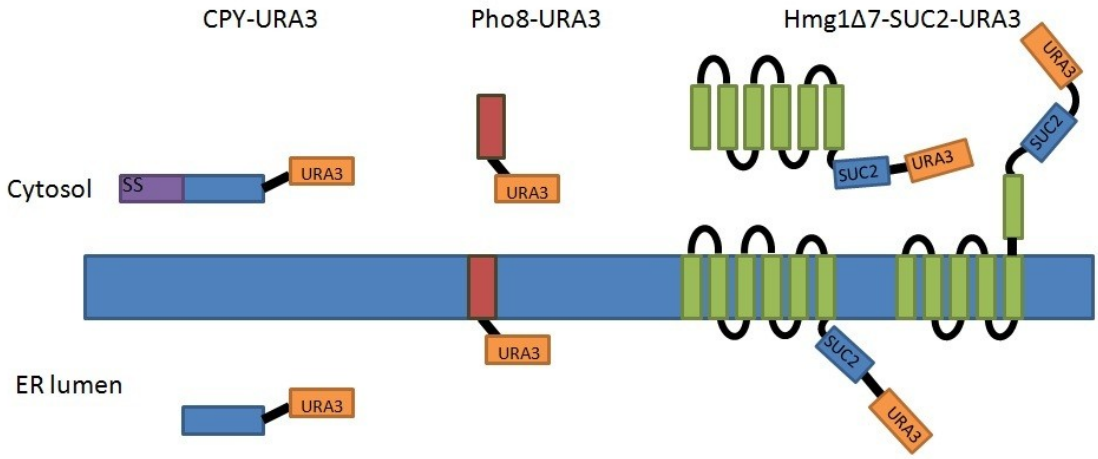


Figure 3.2 Overview of the protein translocation assay. The substrate proteins CPY-URA3, PHO8-URA3 and HMG1Δ7-SUC2-URA3 can exist in different topologies depending on whether they are translocated or integrated correctly. When the proteins are integrated or translocated correctly the URA3 moiety is sequestered in the ER lumen. However, when the proteins are integrated or translocated incorrectly the URA3 moiety localises to the cytosol. When translocated correctly CPY-URA3 is translocated to the ER lumen and its signal sequence (SS) cleaved, PHO8-URA3 is integrated into the ER membrane and lastly, all the transmembrane domains of HMG1Δ7-SUC2-URA3 are integrated into the ER membrane.

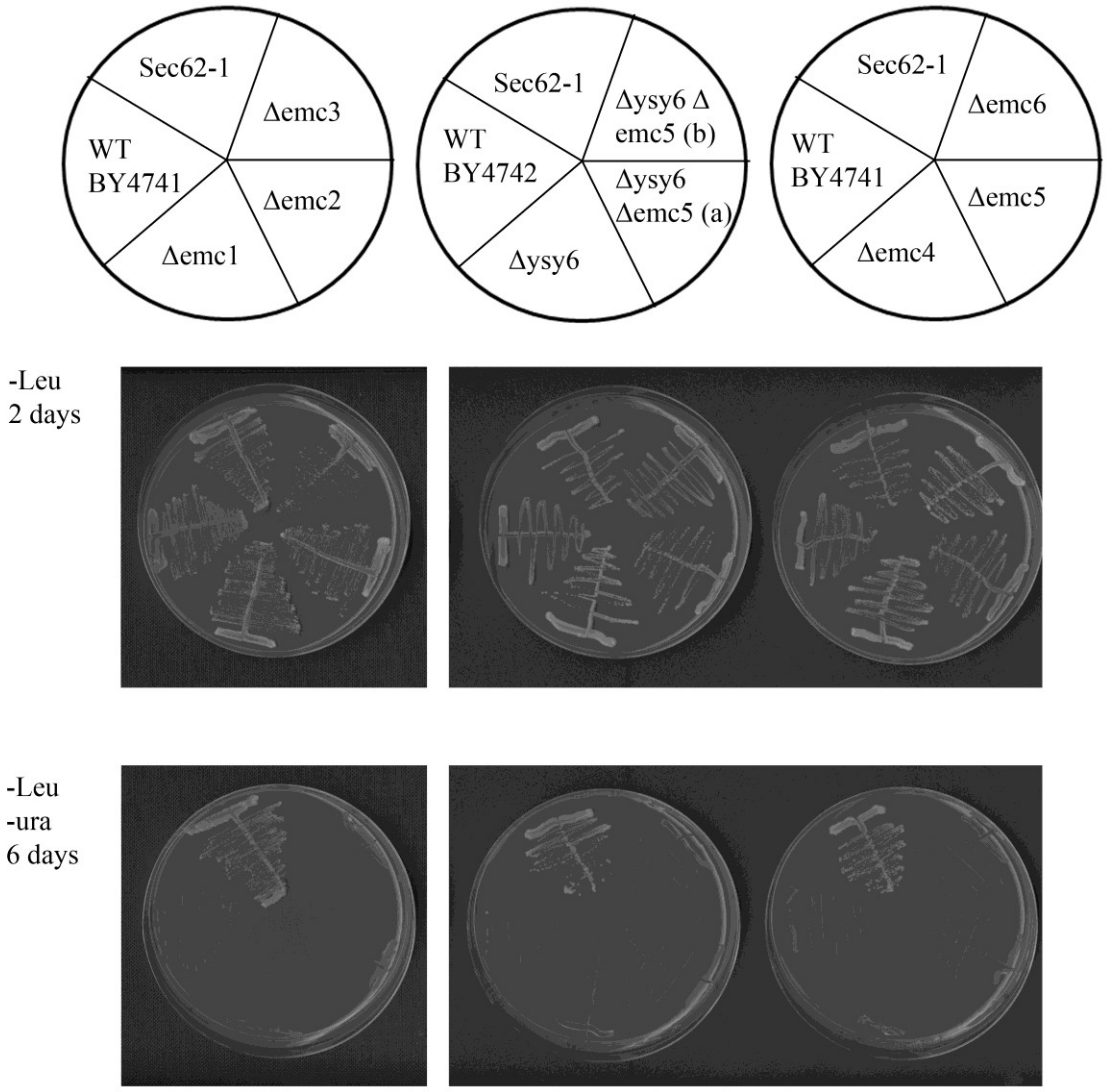


Figure 3.3 Translocation of CPY-URA3 Strains expressing CPY-URA3 were streaked from a freshly grown SD –leu plate onto SD –Leu and SD –Leu -Ura and incubated for the stated number of days at 30°C. The *sec62-1* strain was used as a positive control for defective protein translocation. Figures are representative of 2 repeat experiments.

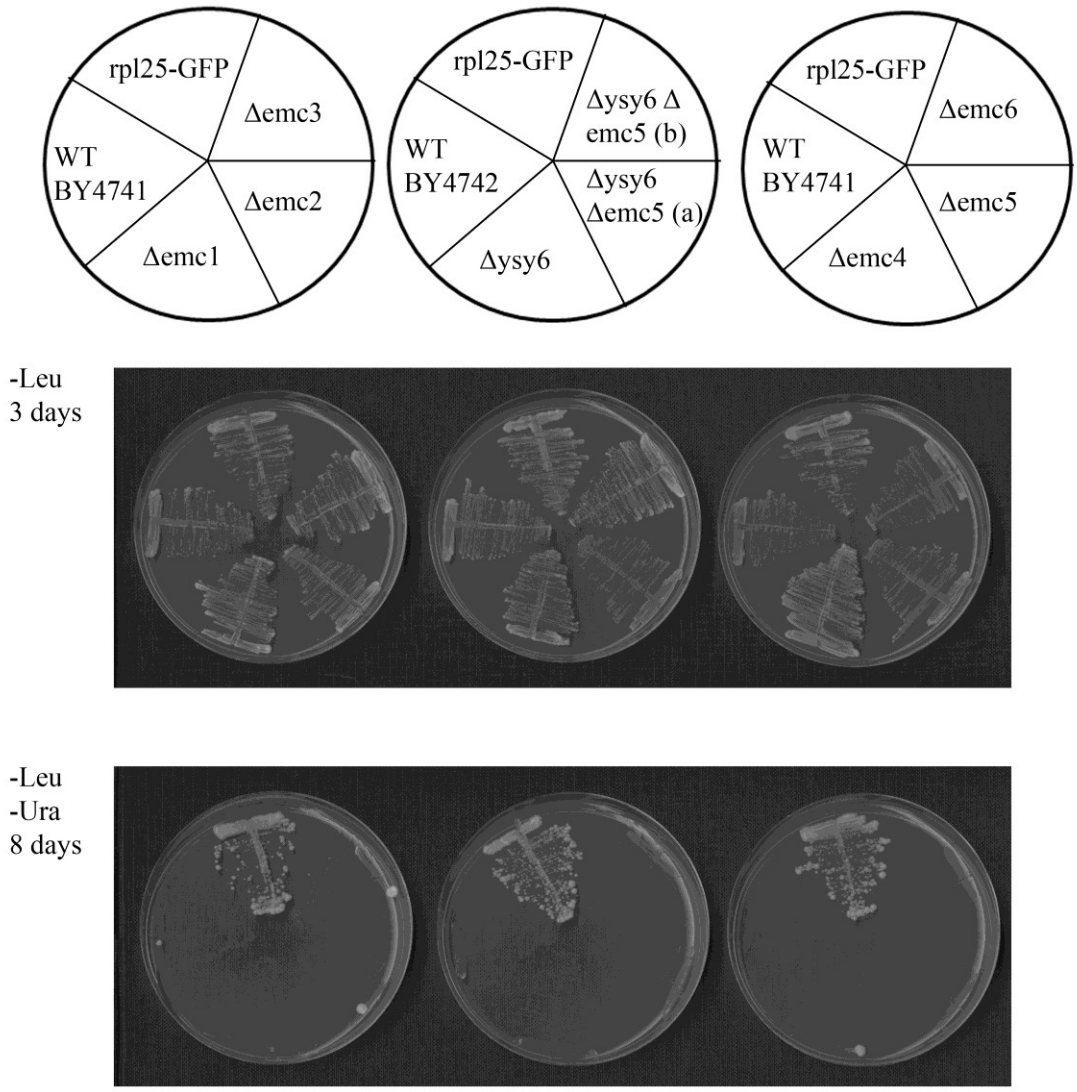


Figure 3.4 Integration of Hmg1Δ7-SUC2-URA3 Strains expressing HMG1Δ7-SUC2-URA3 were streaked from a freshly grown SD –Leu plate onto SD –Leu and SD –Leu -Ura and incubated for the stated number of days at 30°C. The *rpl25-GFP* strain was used as a positive control for defective protein integration. Figures are representative of 2 repeat experiments.

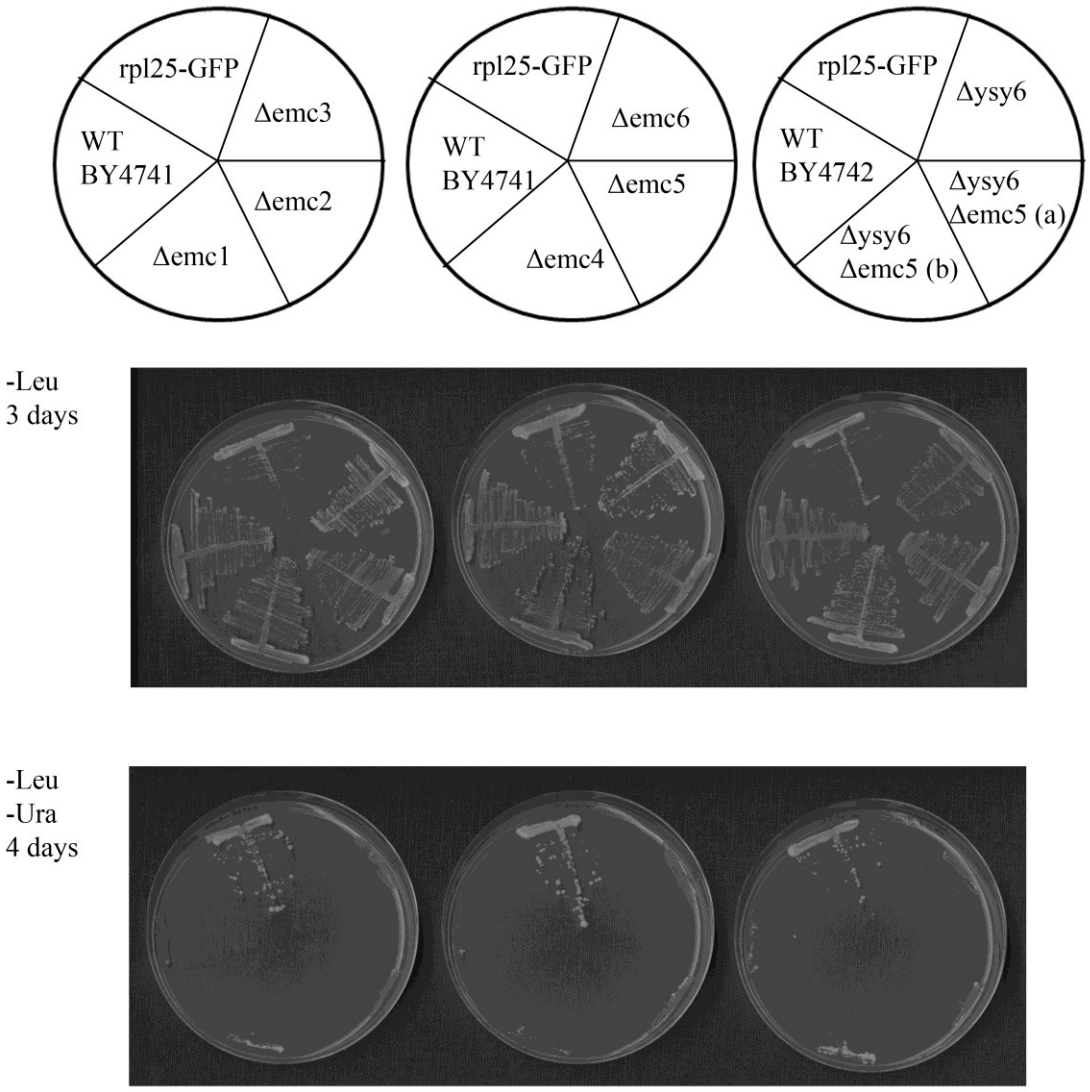


Figure 3.5 Integration of PHO8-URA3 Strains expressing PHO8-URA3 were streaked from a freshly grown SD –Leu plate onto SD –Leu and SD –Leu -Ura and incubated for the stated number of days at 30°C. The *rpl25-GFP* strain was used as a positive control for defective protein integration. Figures are representative of two repeat experiments.

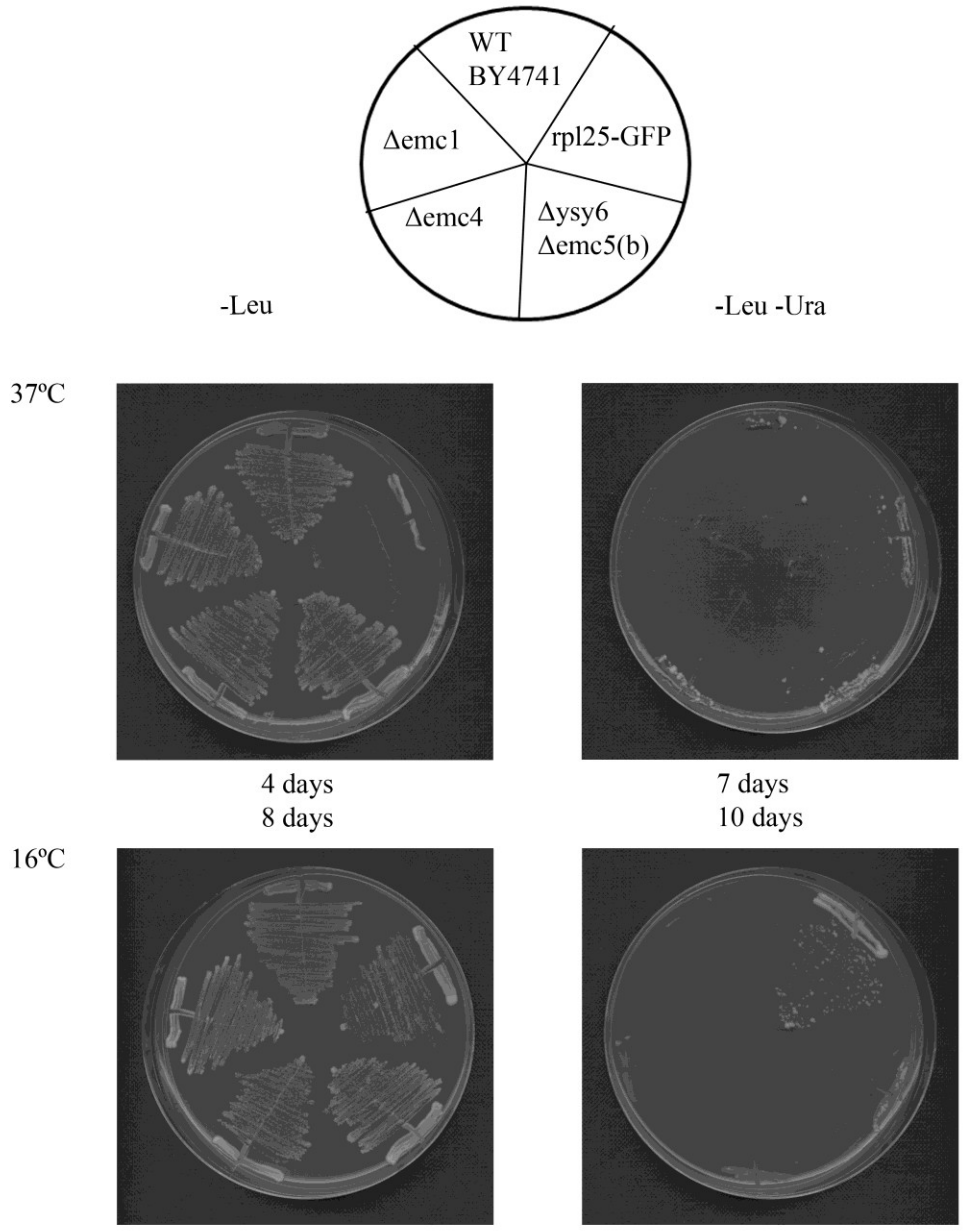


Figure 3.6 integration of PHO8-URA3 at 37°C and 16°C Strains expressing PHO8-URA3 were streaked from a freshly grown SD –leu plate onto SD –Leu and SD –Leu -Ura and incubated for the stated number of days. The *rpl25-GFP* strain was used as a positive control for defective protein integration.

transformed with plasmid encoding CPY-URA3 with leucine selection grew on SD media in the absence of leucine in 2 days, however, in the absence leucine and uracil only the positive control *sec62-1* strain became prototrophic for uracil with strong detectable growth even after 6 days.

The *rpl25-GFP* strain fails to bind SRP efficiently and has been shown to give rise to defects in protein integration specifically for proteins using the SRP dependent co-translational pathway such as Pho8-URA3 and HMG1Δ7-SUC2-URA3 (Dalley et al., 2008). As can be seen in figure 3.4 and 3.5 the positive control *rpl25-GFP* behaved as expected with prototrophy arising for uracil when expressing either PHO8-URA3 or HMG1Δ7-SUC2-URA3. The *rpl25-GFP* strain is slow growing and for this reason the SD –Leu growth control plates were incubated for three days instead of two for the CPY-URA3 assay (Dalley et al., 2008, Hurt et al., 1999). As shown in figure 3.4 no prototrophy could however be observed for HMG1Δ7-SUC2-URA3 in the query strains. As can be seen in figure 3.5 the PHO8-URA3 reporter gave rise to prototrophy in the *rpl25-GFP* strain. No such prototrophy could be observed for query strains expressing PHO8-URA3. However, in figure 3.5 strains Δ*emc1*, Δ*emc4* and the Δ*ysy6*Δ*emc5* (b) strains showed a small amount of growth on the initial streak which was stronger than *WT* growth on media lacking leucine and uracil. To further investigate whether this was indicative of a mild translocation defect the assay was performed at 37°C and 16°C to test whether the phenotype could be enhanced. The slow growing phenotype of the *rpl25-GFP* is exacerbated at 37°C. However, this did not abolish the emergence of protorophy, as shown in figure 3.6 (Hurt et al., 1999). The observed emergence of prototrophy at 30°C in strains Δ*emc1*, Δ*emc4* and the Δ*ysy6emc5* (b) could however not be exacerbated at 37°C or 16°C. In summary, deletion of members of the *EMC* complex and *YSY6* did not give rise to defects in protein translocation and integration for CPY-URA3, Hmg1Δ7-SUC2-URA3 and PHO8-URA3.

3.4 Investigating ERAD in deletion strains of *EMC1*, *EMC5* and *YSY6*

Both Ysy6p and the EMC complex show genetic and physical interactions with components of the ERAD machinery. In order to investigate a potential role for Ysy6p and the EMC complex, the well characterised degradation substrate HMG2-6myc was used. The polytopic membrane protein Hmg2p is one of two isozymes of the 3-

hydroxy-3-methylglutataryl coenzyme A reducatase (HMG-R) (Basson et al., 1986). HMG-R converts Hmg-CoA to mevalonic acid in the biosynthesis of sterols. Hmg2p is rapidly turned over in a manner that is regulated by a feedback mechanism dependent on the level of HMG-R activity (Hampton and Rine, 1994). Degradation of Hmg2p has been shown to occur via the Hrd1p pathway and without the involvement of vacuolar proteases (Hampton et al., 1996, Hampton and Rine, 1994). The 6myc tag in the Hmg2-6myc construct was inserted in a manner which abolishes feedback regulation (Hampton et al., 1996). The cause of the instability of Hmg2p has been shown to be conferred by the properties of its transmembrane domains thereby further demonstrating it to be a *bona fide* ERAD-M substrate (Theesfeld et al., 2011, Carvalho et al., 2006).

The study of degradation of Hmg2-6myc focused on the Δ*ysy6* and Δ*emc5* strains, due to the reported genetic interaction between the *YSY6* and *EMC5* genes (Schuldiner et al., 2005, Ito et al., 2001). Additionally, the Δ*emc1* strain was also investigated as Emc1p is the biggest member of the *EMC* complex and predicted to be a membrane protein with a large soluble domain and was therefore hypothesised to have a central role in the function of the complex (SGD, 2012). SDS and temperature sensitivity assays demonstrated that the Δ*emc1* and Δ*emc5* have differential sensitivity to stress causing agents such as SDS and elevated temperature. The Δ*emc1* is sensitive to both SDS and temperature. However, Δ*emc5* is not sensitive to elevated temperature and only mildly sensitive to SDS (figure 3.1). Studying the ERAD status of both Δ*emc1* and Δ*emc5* can therefore shed more light as to whether any defect in ERAD can be correlated to growth defects at elevated temperature or on media containing SDS.

Degradation of Hmg2-6myc has been previously characterised by cycloheximide chase (Hampton et al., 1996). Cycloheximide blocks protein synthesis by blocking peptidyl tRNA from moving from the ribosomal A to the P site thereby allowing the degradation of previously sysnthesised protein to be monitored (Obrig et al., 1971). The degradation of Hmg2-6myc in Δ*emc1*, Δ*emc5* and Δ*ysy6* strains was therefore investigated by cycloheximide chase. Cells were propagated overnight in selective SD media followed by propagation for two cell doublings in rich liquid YPD media. Cycloheximide was then added to the media and the first sample T=0 was taken two minutes after cycloheximide addition. The chase was carried out for 180 minutes and repeated three times for *WT*, Δ*emc1,* Δ*emc5* and Δ*ysy6* strains. Deletion of *HRD1* causes an almost

complete stabilisation of Hmg2-6myc and therefore a Δ*hrd1* strain was used as a positive control (Hampton et al., 1996). At the end of the chase, cells were collected and lysed with sodium hydroxide followed by TCA precipitation and resuspension in sample buffer. Repeats for each strain were loaded onto 8% SDS-PAGE gels and blotted concomitantly with anti myc antibody followed reprobing of the same nitrocellulose membranes with anti-Zwf1p antibody. Reprobing of the same SDS-PAGE gel ensured that the relative anti-Zwf1p and anit-myc antibody signals were not affected by inaccuracies in sample loading onto different SDS-PAGE gels.

Specificity of the anti-myc antibody is shown in figure 3.7(a). As can be seen the myc antibody gave rise to the expected single strong band of 140 kDa for Hmg2-6myc (Hampton et al 1996). Cells not expressing Hmg2-6myc or expressing Ssh1-myc were used as negative and positive controls respectively.

Representative blots of cycloheximide chase experiments are shown in Figure 3.7 (b). As can be seen the positive control Δ*hrd1* strain showed strong stabilisation of Hmg2-6myc. However, strains Δ*emc1*, Δ*emc5* and Δ*ysy6* did not give rise to strong stabilisation of Hmg2-6myc. However, a certain degree of weak stabilisation could be observed in the Δ*ysy6* and Δ*emc5* strains.

In order to further investigate the degradation profile the relative amount of Hmg2-6myc was quantified. Since SDS-PAGE, transfer and blotting were carried out concomitantly for each strain, degradation curves could be constructed. The amount of Hmg2-6myc and Zwf1p was quantified using Aida by drawing boxes of identical sizes for each band. Background was removed by establishing the threshold for detection of background. This threshold was then applied to all boxes within a blot. The total myc signal values were then divided by the total Zwf1p signal values and then normalised to give rise to T_0=1 and subsequent time points then expressed as a fraction of T_0. Degradation curves were constructed using Prism using a one-phase exponential decay model. Curves constructed for the *WT,* Δ*emc5, Δysy6* and Δ*emc1* strains gave rise to values of goodness of fit (R squared values) of 0.915, 0.766, 0.723 and 0.894 respectively (Colin Cameron and Windmeijer, 1997). The *WT* strain showed a degradation half life of Hmg2-6myc of 44 minutes which consistent with previous observations of a half life of 0.5-1h (Hampton et al., 1996). As can be seen in figure 3.8 the *Δemc1* deletion strain shows no defect in degradation of Hmg2-6myc, with a profile

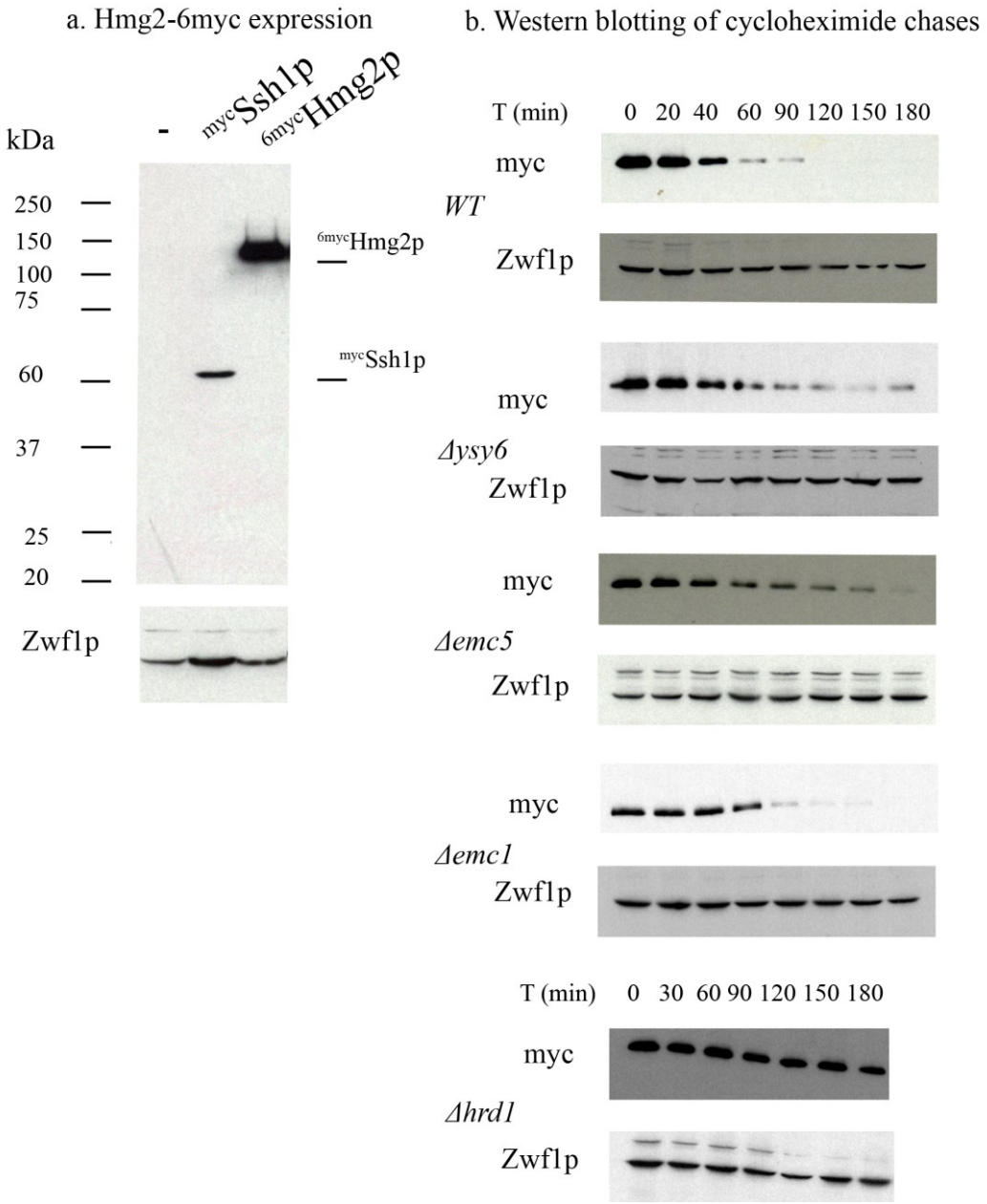


Figure 3.7 Western blotting of cycloheximide chase of Hmg2-6myc. (a) The specificity of the anti myc antibody was characterised by propagating the untransformed *WT* strain, the *WT* strain transformed with pR16 expressing Ssh1-myc and the *WT* strain transformed with pRH244 expressing Hmg2-6myc in appropriate minimal SD media to stationary phase followed by propagation in YPD media to mid log phase. 3 OD_{600} units of cell culture were then lysed by NaOH lysis and the protein TCA precipitated. Samples were then analysed by loading 0.1 OD_{600} on an 8% SDS-PAGE gel followed by western blotting with anti-myc and anti Zwf1p antibody. (b) Indicated strains transformed with pRH244 were propagated in SD – Ura media to stationary phase. Strains were then propagated in YPD media to log phase and cycloheximide added to a final concentration of 0.5 mg/ml. After addition of cycloheximide 3 OD_{600} units of cell culture were collected at the indicated times and treated with 10 mM azide on ice followed by cell lysis by NaOH lysis and TCA precipitation of protein. Samples were then analysed by loading 0.1 OD_{600} on 8% SDS-PAGE gels and western blotting using the anti myc and anti Zwf1p antibody. Figures are representative of three repeat experiments.

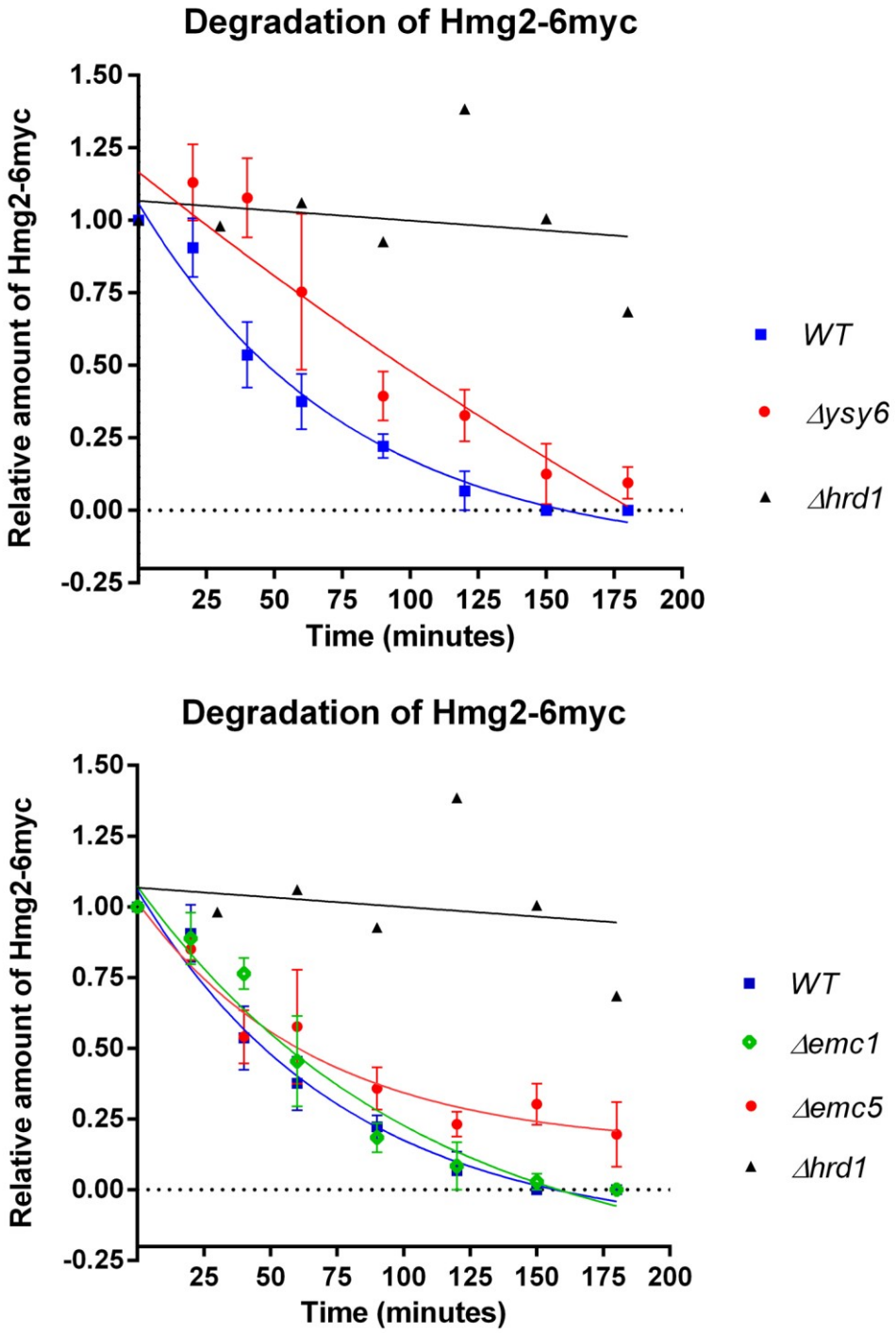


Figure 3.8 Quantification of cycloheximide chase experiments. Western blots were quantified using AIDA image analyzer. The Hmg2-6myc signal was normalised to the loading control, Zwf1p. The T=0 sample was then normalised to 1 and subsequent dilutions expressed in terms of relative quantity thereof. Error bars +/- standard error of the mean from three repeat experiments.

indistinguishable from the *WT* strain. However, the Δ*emc5* strain shows that clearance of Hmg2-6myc becomes impaired as Hmg2-6myc levels decrease with no additional significant degradation after T=90. This therefore demonstrates that the Δ*emc5* strain shows impaired degradation of the ERAD-M substrate Hmg2-6myc. The *Δysy6* strain showed no defect in degradation of Hmg2-6myc as clearance of Hmg2-6myc occurred concomitantly with the *WT* strains. However, it is clear that the *Δysy6* shows an altered degradation profile with elevated amounts of Hmg2-6myc relative to *WT* at time points T=40 to T=120. In addition, no significant degradation of Hmg2-6myc could be observed between time point T=0 and T=60. Therefore the degradation profile appears biphasic with initial stabilisation of Hmg2-6myc followed by initiation of more rapid degradation. The graphical observations of altered degradation of Hmg2-6myc in the *Δysy6* and Δ*emc5* strains is mirrored in the R-squared values which demonstrate that strains unimpaired in degradation of Hmg2-6myc are in tighter agreement with the one phase exponential decay model than the strains which show altered degradation of Hmg2-6myc. The data therefore demonstrate, firstly, that both *Δemc5* and *Δysy6* but not *Δemc1* have altered degradation of Hmg2-6myc and therefore affect ERAD-M. Secondly, that the SDS and temperature sensitivity phenotypes are not concomitant with defects in ERAD as the *Δemc1* strain which is sensitive to both SDS and elevated temperature shows no alteration in the degradation of Hmg2-6myc.

3.5 Investigating Trafficking in deletion strains of *EMC5* and *YSY6*

It has recently been demonstrated that the EMC complex shows defective forward trafficking of the polytopic plasma membrane protein Mrh1p-GFP (Bircham et al., 2011, Wu et al., 2000). In deletion strains of the *EMC* complex a pool of Mrh1p-GFP is shown to be mislocalised to the ER. The function and trafficking of Yor1p and Yor1p-GFP constructs have been previously characterised (Pagant et al., 2008, Pagant et al., 2007, Epping and Moye-Rowley, 2002, Katzmann et al., 1999, Katzmann et al., 1995). Therefore, a similar Yor1p-GFP C terminal fusion was used to further investigate protein trafficking. Yor1p is an ABC transporter and therefore a polytopic membrane protein which localises to the plasma membrane and vacuole and confers resistance to oligomycin (Katzmann et al., 1999, Katzmann et al., 1995). Modification of various domains of Yor1p-GFP and Yor1p have been shown to affect protein stability and localisation which, for example, can cause ER retention (Pagant et al., 2008).

Furthermore, it has been suggested that multiple signals are required for forward trafficking of Yor1p-GFP (Epping and Moye-Rowley, 2002). One of these signals has been shown to be the B-site acceptor motif DxE of Yor1p which promotes forward trafficking by the COPII vesicle trafficking machinery (Pagant et al., 2007). However, ER retention of Yor1p-GFP has been shown to be due to folding defects as well as trafficking defects (Pagant et al., 2008, Pagant et al., 2007). Investigating the localisation of Yor1p-GFP will shed light firstly on the status of the ER quality control and COPII trafficking machinery and secondly as to whether the mis-localisation of the polytopic protein Mrh1p-GFP previously reported by Bircham et al., (2011) is a general feature of deletion of *EMC5*.

Cells overexpressing Yor1-GFP under the control of the methionine repressible *MET25* promoter were propagated to exponential phase in SD media lacking methionine and uracil and visualised by live cell microscopy. As can be seen in figure 3.9 cultures displayed a range of expression levels of the substrate. Yor1p-GFP was shown to localise to the plasma membrane and intracellularly as described previously by Epping and Moye-Rowley (2002) in the *WT* strain. However, in addition punctuate structures could be observed in certain *WT* cells. The *BY4742 Δysy6* and *Δemc5* strains showed similar localisation. Perinuclear staining is indicative of ER retention, however, in no instance was perinuclear staining observed as previously reported for Yor1p-GFP mutants (Pagant et al., 2008, Pagant et al., 2007, Huh et al., 2003, Epping and Moye-Rowley, 2002). The *Δysy6* strain appears here to have cells with an elevated number of puncta relative to the *WT* strain (figure 3.9). In order to determine whether this was a representative phenotype of the *Δysy6* strain the number of cells expressing Yor1-GFP were counted in fields of view across three repeat experiments. The data from each strain was tested for normality by the Kolmogorov-smirnov and Shaipro test using IBM® SPSS® v20. This showed that the data were not normally distributed and therefore differences between data sets were compared using the Mann-Whitney *U* test. This showed that the number of puncta per cell were not significantly different between the *WT* and *Δysy6* strains and the *WT* and *Δemc5* strains, as shown in figure 3.10. Therefore, no defect in localisation of Yor1p-GFP could be observed in the *Δysy6 and Δemc5* strains.

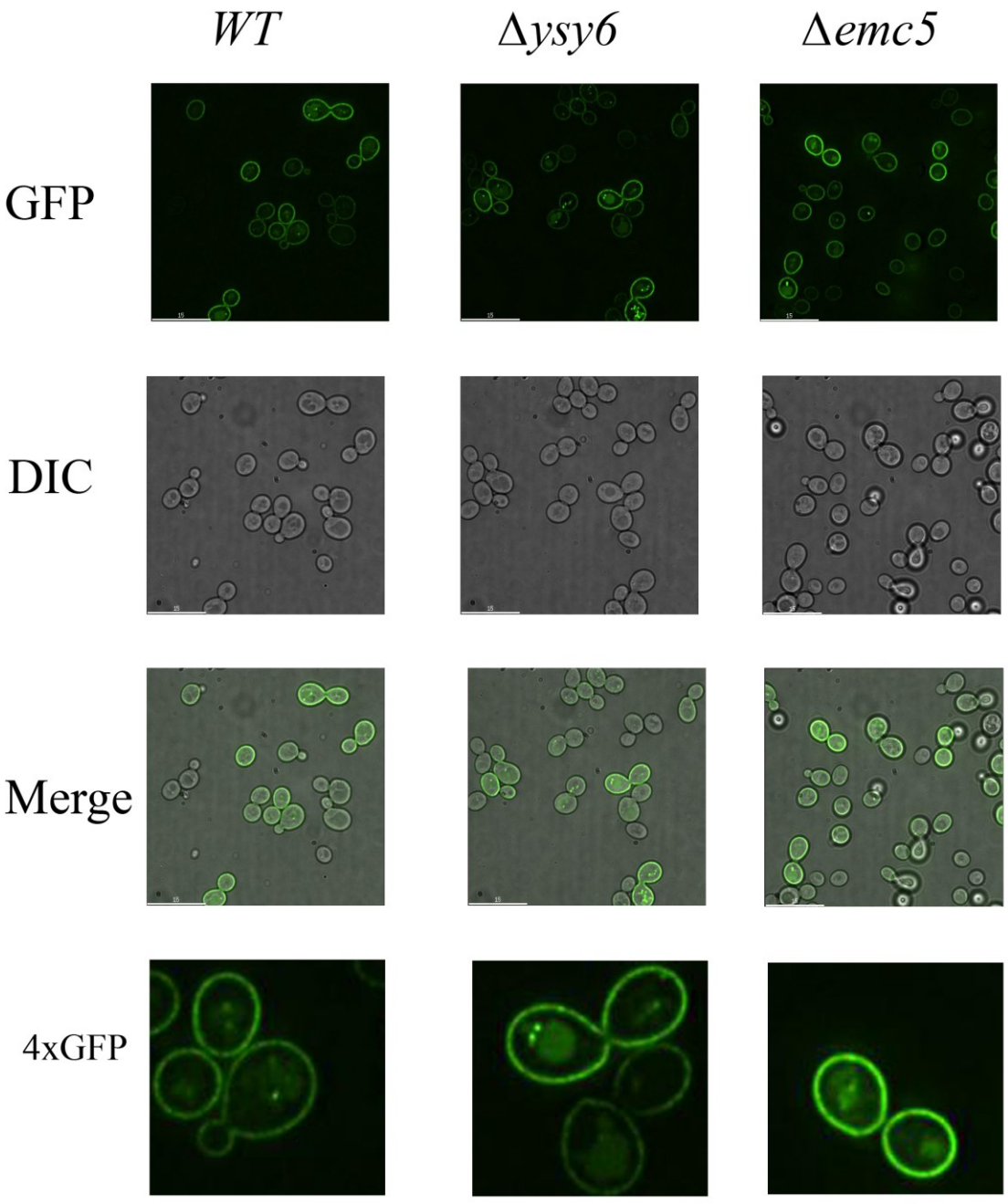


Figure 3.9 Localisation of Yor1p-GFP Cells expressing Yor1-GFP under the control of the MET25 promoter were grown to log phase in selective –Ura–Met SD media and concentrated 50 fold by centrifugation at 1 200 x g and visualised by live cell imaging. Images taken using transmitted light field (DIC) and FITC filter (GFP). Cells from the FITC field (GFP) are also presented enlarged 4x (4xGFP).

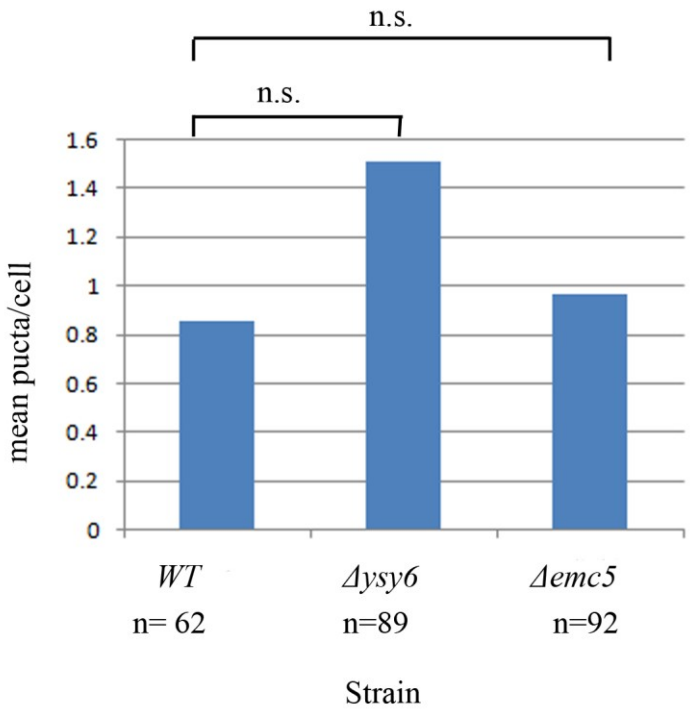


Figure 3.10 The number of punctuate structures is not significantly different between strains. The number of punctate structures per cell were counted in the indicated number of cells (n) expressing Yor1p-GFP in fields of view from three repeat experiments. Normality testing performed with SPSS for independent variables showed that the data is not normally distributed. Therefore Mann-Whitney *U* tests for significance were carried out and showed that the strains were not significantly different (ns) in average number of puncta.

3.6 Discussion

The effect of deleting members of the EMC complex and YSY6 was invstigated by assessing the status of the translocation, forward trafficking and ERAD machinery. In addition the relative growth of the strains was assessed at various temperatures and on media containing SDS. The *Δysy6* strain showed no defect in protein integration for co-translational and post-translational substrates and for both lumenal and transmembrane substrates tested. In addition, Yor1p-GFP is not retained in the ER which suggests that both the ERQC and the COPII forward trafficking machinery are not compromised by deletion of *YSY6* (Pagant et al., 2007). Lastly, *YSY6* and its mammalian homolog, *RAMP4*, have been shown to be implicated with stress responses. Deletion of *RAMP4* was shown to upregulate stress markers GRP78 and GRP94 whereas deletion of *YSY6* causes UPR induction (Jonikas et al., 2009, Hori et al., 2006). In order to investigate a possible role for *YSY6* in stress responses relative growth of a *Δysy6* strain was investigated in stress inducing conditions such as growth at low or high temperature or on media containing SDS (Corcoles-Saez et al., 2012, Scrimale et al., 2009, Leber et al., 2004, Trott and Morano, 2003, Bickle et al., 1998). The *Δysy6* showed no defect in growth at temperatures between 16 and 39.5°C or on media containing SDS. This suggests that *YSY6* is not required for survival under the tested stress inducing conditions.

Deletion strains for the *EMC* complex members also showed no defect in protein integration for the substrates tested. Deletion strains of the *EMC* complex showed no defect for growth at temperatures between 16 and 37°C. The *Δemc5* strain showed a mild defect in growth on media containing SDS and the *Δemc2* strain showed defects in growth at elevated temperature of 39.5°C. Other members of the *EMC* complex showed defects for both growth on media containing SDS and growth at 39.5°C for strains *Δemc1*, *Δemc3* and *Δemc6*. This clearly demonstrates that the EMC complex is involved in survival to stress.

Both SDS and elevated temperature induce cell wall stress (Scrimale et al., 2009, Bickle et al., 1998). SDS sensitivity suggests that these strains have a defect in cell wall biogenesis which can be caused by upstream components of the secretory pathway (Bickle et al., 1998). This is consistent with previous work which has shown that the *EMC* complex is localised to the ER and thought to be involved in protein biogenesis or ERAD (Christianson et al., 2012, Jonikas et al., 2009). In addition, it is known that

members of the EMC complex in both yeast and mammals are upregulated during the UPR (Christianson et al., 2012, Travers et al., 2000). It is therefore reasonable to assume that these genes have a function in the UPR. It is known that both SDS and temperature above 37°C can activate the UPR (Scrimale et al., 2009, Leber et al., 2004). However, since strains *Δemc1*, *Δemc2, Δemc3* and *Δemc6* do not show growth defects at 37°C this suggests that the UPR needs to be strongly induced before these genes become essential or required for survival. This suggests that the *EMC1*, *EMC2*, *EMC3*, *EMC6* genes are required for survival under conditions of extreme stress. However an alternative hypothesis is that deletion of these genes renders the EMC complex temperature sensitive by destabilising the complex. This still suggests that the EMC complex as a functional entity is required in conditions of elevated stress. Supporting this is the observation that in the presence of the UPR inducing agent, tunicamycin, *Δemc1*, *Δemc3* and *Δemc5* complex members becomes lethal in the absence of *HAC1* (Bircham et al., 2011). Tunicamycin prevents N-linked glycosylation and therefore impedes protein folding (Varki A, 1999). In the work carried out here it was seen that deletion strains *Δemc1*, *Δemc3* and *Δemc6* are defective in growth under conditions which induce UPR stress. This discrepancy may be due to the exact nature of the stress. Elevated temperature, for example, is known to induce both the UPR and Heat shock response (Leber et al., 2004, Trott and Morano, 2003). KAR2 transcription for example is regulated by both pathways independently (Trott and Morano, 2003, Mori et al., 1992). It can therefore not be excluded that expression of members of the EMC is regulated by multiple pathways or differentially required depending on the nature of the stress.

The *Δysy6* and *Δemc5* strains showed no defect in growth at differential temperature. However, the *Δysy6* and *Δemc5* double deletions strains (a) and (b) showed discrepancy in SDS sensitivity with strain (a) having an increased sensitivity to SDS and (b) a decreased sensitivity to SDS. It can therefore not be determined whether the double deletion is neutral or aggravating in relation to the single deletions. However, neither of these strains have defects in protein integration showing that irrespective of SDS sensitivity status the strains show no defect in protein integration. This therefore demonstrates that double deletion of *Δysy6* and *Δemc5* does not lead to defects in protein integration for the substrates tested and suggests that the protein integration machinery is not responsible for the previously reported interactions.

Both the *Δysy6* and *Δemc5* strains showed altered degradation of the Hmg2-6myc substrate. The *Δysy6* strain shows initial stabilisation of the degradation substrate followed by rapid degradation. This suggests that at least two mechanisms of degradation are involved. Firstly, the deletion of *Δysy6* causes a defect in degradation via the Hrd1p dependent pathway. Secondly, that an alternative pathway of degradation is being used after prolonged exposure to cycloheximide. Cycloheximide exposure has been demonstrated to activate the UPR and cause the elicitation of alternative degradation pathways for an ER localised substrate in mammalian cells (Shenkman et al., 2007). In addition, it is also known that the UPR can induce vacuolar degradation of CPY* in yeast (Spear and Ng, 2003). Strikingly, it was shown that CPY* degradation was inhibited in cycloheximide chase assays when vacuolar degradation was compromised. However, the Δ*hrd1* positive control strain does not show elicitation of an alternative degradation pathway. This suggests that elicitation of this second pathway is attributable to loss of function of Ysy6p and therefore that Ysy6p functions as a direct or indirect regulatory component of the protein degradation machinery.

The *Δemc1* strain shows no defect in ERAD. However, the *Δemc5* strain showed a defect in degradation of the ERAD substrate Hmg2-6myc. This suggests that deletion of *EMC5* results in defective ERAD for the Hrd1p dependent pathway. However, the ERAD defect in the *Δemc5* strain only becomes apparent after significant clearance of Hmg2-6myc substrate has occurred at T=90. From this point the Hmg2-6myc substrate shows no significant additional degradation. This observation therefore suggests that the defect in ERAD may be due to a pool of Hmg2-6myc which is protected from degradation. This pool is no longer accessible for ERAD possibly due to depletion of factors required for ERAD. However, the identity of these cofactors would remain elusive. Alternatively, it is possible that the ERAD defect is actually the result of a defect in retention of Hmg2-6myc which would suggest a role of the EMC complex in ER-golgi trafficking.

The EMC complex has previously been demonstrated to be involved in trafficking as it has been reported that deletions in members of the *EMC* complex give rise to ER retention of the substrate Mrh1p-GFP (Bircham et al., 2011). However, deletion of *EMC5* does not result in ER retention of Yor1-GFP. This therefore demonstrates that ER retention is substrate dependent. Consistent with this is that Bircham et al., (2011) could not see any defects in processing in CPY, demonstrating that trafficking of CPY

remains unaffected. However, the authors suggest that the EMC complex is involved in the trafficking of membrane proteins and also report interactions of the EMC complex with ERAD-C factors. However, data presented here clearly shows a defect in ERAD M. In summary, the *Δemc5* strain shows a defect in the clearance of Hmg2-6myc which could result from either defective ERAD function or upstream trafficking pathways.

The *EMC* complex has therefore been involved with stress and ERAD. However, only the function of *EMC5* has been implicated in ERAD-M in this study whereas *EMC1* deletion has been shown not to affect Hmg2-6myc. It would therefore be of interest to establish the extent of the involvement of the EMC complex in ERAD. In order to investigate this, deletion strains of other members of the *EMC* complex should be investigated using the Hmg2-6myc substrate. Secondly, the scope of the ERAD defects should be investigated. This can be done by investigating other classes of substrates such as the ERAD-C substrate Ste1-166 and the ERAD-L substrate CPY* (Vashist and Ng, 2004, Swanson et al., 2001). In addition, with the knowledge that the EMC complex is involved in stress responses it can be investigated whether either ERAD or protein integration processes are affected or further affected under conditions of stress. This could be investigated by performing ERAD studies following induction of stress responses. Protein integration studies under conditions of stress studies can be carried out using by western blotting or immunoprecipitation of radiolabled proteins of cell lysates from strains grown under conditions of cellular stress for processed and glycosylated reporters such as Hmg1Δ7-SUC2-URA3 or CPY (Bircham et al., 2011, Stirling et al., 1992). Lastly, in order to investigate whether the ERAD defect is a result of a defect in retention, localisation of Hmg2-6myc can be carried out microscopically.

The *YSY6* deletion strain showed an ERAD defect which was hypothesied to be due to a defect in the elicitation of an alternative pathway. Firstly, to investigate whether the effect is due to cycloheximide the assay can be repeated using a pulse chase approach which does not require the use of cycloheximide. Secondly, it was hypothesised that Ysy6p may regulate substrate delivery to the Hrd1p complex. Yamaguchi et al., (1999) demonstrated that overexpression of the mammalian homolog of Ysy6p, RAMP4, protected proteins from degradation. It can therefore be investigated if overexpression of Ysy6p has a similar effect in the degradation of Hmg2-6myc. It has also been proposed that deletion of Hrd3p causes loss of selectivity of ERAD substrates when Hrd1p is overexpressed (Denic et al., 2006). Therefore it can be investigated whether

the double deletion of *HRD3* and *YSY6* over-expressing Hrd1p has an alleviating effect on ERAD relative to the deletion of *HRD3* over-expressing Hrd1p.

In summary it has been shown that deletion of *EMC1, EMC2*, *EMC3* or *EMC6* causes defects in growth under conditions which cause stress. It has also been shown that the *EMC5* strain shows a defect in ERAD-M of Hmg2-6myc. Intrestingly it has been shown that the ERAD and stress sensitivity phenotypes are distinct due to the fact that deletion of *EMC1* gives rise to a stress sensitivity phenotype but no defect in ERAD but that deletion of *EMC5* causes a defect in ERAD but no growth defects. This suggests that distinct members of the *EMC* complex are required for the function of distinct processes. In addition it has been shown that deletion of *YSY6* causes an alteration in the degradation of Hmg2-6myc. This altered degradation appears biphasic and may be indicative of the induction of alternative degradation pathways. The data therefore suggest that Ysy6p functions in ERAD-M.

CHAPTER 4: Ribosome association of Ysy6p

4.1 Introduction

The mammalian homolog of Ysy6p, RAMP4, was identified by Görlich and Rapoport (1993) as a protein which cosediments with ribosomes after solubilisation of the ER. In addition, the proportion of total RAMP4 which cosediments with ribosomes was shown to be of approximately 10-20% (Pool 2009). Furthermore, a small amount of RAMP4 was shown to co-purify with the Sec61 complex after removal of ribosomes. Subsequent work showed that RAMP4 coimmunoprecipitates with Sec61β and Sec61α and crosslinks to Sec61β (Yamaguchi et al., 1999). It has therefore been shown that RAMP4 is a ribosome-associated membrane protein (RAMP) with close interaction with the Sec61p complex. Further evidence of this close interaction is demonstrated by the fact that, during co-translational translocation, the nascent chain can be crosslinked to RAMP4 (Schroder et al., 1999). Taken together the literature provides good evidence that RAMP4 is involved in protein biogenesis and closely associated to the Sec61 complex. Furthermore, in general, ribosome associated membrane proteins (RAMPS) are associated with protein biogenesis (Antonin et al., 2000, Menetret et al., 2000, Meyer et al., 2000, Wang and Dobberstein, 1999, Gorlich et al., 1992a, Gorlich et al., 1992b). It is currently unknown whether Ysy6p is ribosomose associated. Therefore investigating whether Ysy6p is a RAMP will in turn shed light onto whether Ysy6p is implicated in protein biogenesis and whether Ysy6p is a *bona fide* homolog of RAMP4.

A variety of methods exist for identifying ribosome associated proteins. The most commonly used methods include ribosome sedimentation through a cushion or gradient, affinity purification or ribosome salt washes and mass spectrometry (Halbeisen et al., 2009, Fleischer et al., 2006, Frey et al., 2001, Chevet et al., 1999). In this study the use of sedimentation through sucrose cushions and gradients was chosen over the use of salt washes and mass spectrometry. The main reason for this is the fact that centrifugation through a sucrose cushion is the method which involves the smallest number of steps and therefore minimises the risk of protein degradation. Secondly, this study is focused on Ysy6p whereas methods involving ribosome affinity purification and mass spectrometry are better suited for identifying completely novel factors (Halbeisen et al., 2009, Fleischer et al., 2006). The main difficulty in assessing ribosome association is defining the specificity of any protein interaction to ribosomes as ribosomes are considered to be inherently “sticky” (Serebriiskii et al., 2000). In

molecular terms, the term “sticky” is rationalised by the fact that the ribosome surface is negatively charged and therefore proteins can non-specifically associate with the ribosome by electrostatic interactions. This is however overcome by using High salt buffers (Gorlich et al., 1992b).

4.2 Ysy6p cosediments with ribosomes

The cosedimentation assay selectively sediments high molecular complexes such as ribosomes after solubilisation of the ER (Frey et al., 2001). The methodology is summarised in Figure 4.1. Cells were grown to mid log phase and treated with cycloheximide which causes translational arrest (Obrig et al., 1971). The cells were lysed by glass bead disruption and unlysed cells and cell wall debris were sedimented at low speed and the supernatant taken. The lysate was then solubilised with detergent and the unsolubilised material sedimented by centrifugation at 16 000 x g. The 16 000 x g supernatant was then centrifuged over a sucrose cushion at 200 000 x g to sediment the ribosomes. The sucrose cushion acted as an intermediate layer between the supernatant and the sedimented material and therefore enabled a cleaner separation of the two fractions.

The assay requires that membranes are solubilised without disruption of the complex being investigated. Different protein complexes are more or less resistant to solubilisation with different detergents. This property has been used to help identify complexes by categorising them as strongly resistant to detergent or weakly resistant to detergent using detergents of various strengths (Christianson et al., 2012). In the example cited the strong detergent used was Triton X-100 and the weak detergent was Digitonin.

It has already been shown that the association of mammalian Sec61p with ribosomes is dependent on solubilisation with Digitonin but abolished with Triton X-100 (Chevet et al., 1999, Gorlich et al., 1992b). For this reason a detergent with similar properties to Digitonin was chosen that has already been demonstrated not to abolish binding of Sec61p to ribosomes and to maintain association with ribosome associated membrane proteins (Nazari et al., 2012, Chevet et al., 1999, Panzner et al., 1995, Gorlich et al., 1992b). The detergent chosen was CHAPS.

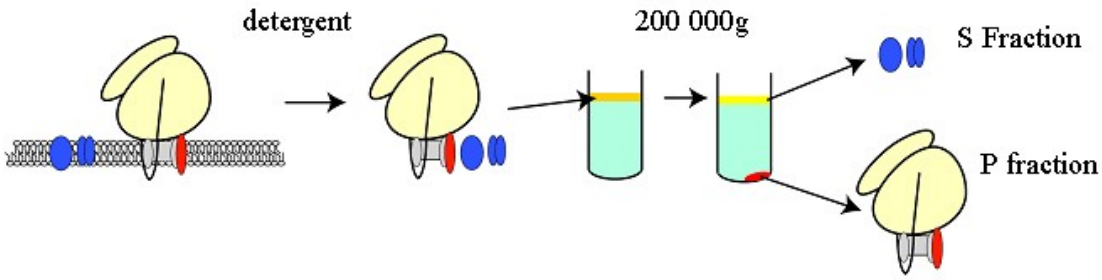


Figure 4.1 Overview of the ribosome cosedimentation assay Cells are lysed in 500 mM Potassium acetate high salt buffer followed by solubilistation in detergent. The solubilised lysate is then centrifuged at 16000 x g for 20 minutes prior to centrifugation over a sucrose cushion at 200 000 x g. This then gives rise to a pellet (P) containing sedimented ribosomes and a supernatant fraction (S) containing soluble material.

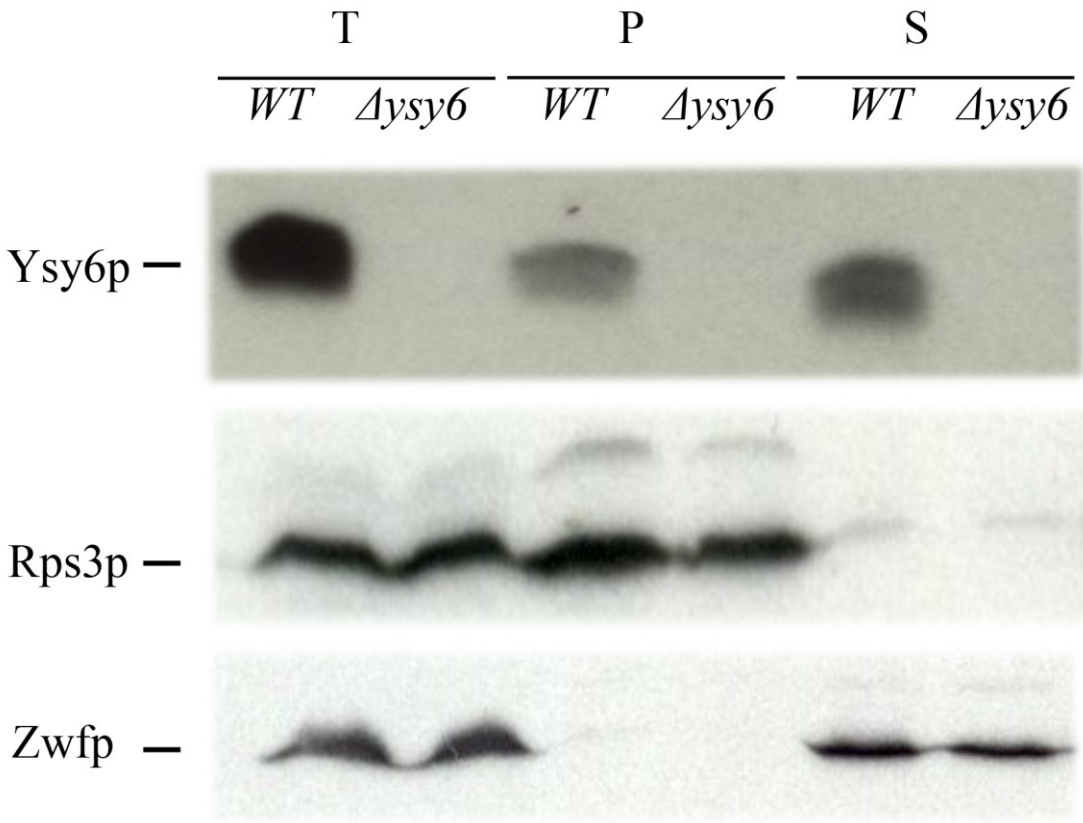


Figure 4.2 Ysy6p cosediments with ribosomes *BY4742 WT* and *Δysy6* strains were propagated in YPD media, lysed in HS buffer and solubilised in 3% (w/v) CHAPS, 10% (v/v) glycerol and sedimented through a high salt cushion. Total (T), Pellet (P) and supernatant (S) fractions were methanol precipitated prior to analysis by SDS-PAGE. 2 OD_{600} of cell culture equivalent were loaded onto a 15% SDS-PAGE gel for western blotting with Ysy6p antibody and 1 OD_{600} of cell culture equivalent was loaded onto a 10% SDS-PAGE gel for western blotting with Rps3p and Zwf1p antibody. Representative of 2 repeat experiments

As shown in figure 4.2 a pool of Ysy6p was found to be present in the P (pellet) fraction as could the ribosomal protein Rps3p but not the cytosolic protein Zwf1p. A protein of the 40S ribosomal subunit, Rps3p was used as a marker for sedimentation of ribosomes (Frey et al., 2001). Zwf1p is used as a negative control for cytosolic contamination of the P fraction (Frey et al., 2001). Ysy6p therefore could be co-sedimented with ribosomes. This result was observed after optimisation of solubilisation conditions using CHAPS to a final concentration of 3 % (w/v) and the use of glycerol to a final concentration of 10% (v/v). Glycerol was added to help avoid protein aggregation (Gutmann et al., 2007)

In order to further investigate the physiological relevance of the co-sedimented portion of Ysy6p the experiment was repeated using additional controls. The ribosome associated protein Sec61p was used as a positive control for physiologically relevant co-sedimentation (Görlich and Rapoport, 1993). In addition, Pho8p was used as a negative control for ribosome co-sedimentation. Pho8p is a transmembrane alkaline phosphatase which localises to the vacuole after glycosylation in the ER and Golgi (Klionsky and Emr, 1989). Pho8p is therefore trafficked to a spatially distinct area from that of ER bound ribosomes and is not known to associate with ribosomes.

As demonstrated in figure 4.3 ribosomes were efficiently sedimented as demonstrated by the Rps3p signal being found strongly enriched in the pellet fraction with trace levels in the supernatant. The cytosolic protein Zwf1p was found exclusively in the supernatant fraction demonstrating that the pellet is not contaminated by cytosolic material. Pho8p was expected to be present exclusively in the supernatant but was also found at trace levels in the pellet (P) fraction. It has previously been shown that 20-30% of Sec61p is ribosome associated (Görlich and Rapoport, 1993, Panzner et al., 1995). Here Sec61p cosedimented to a degree which is consistent with this previous observation.

It was noted that the Ysy6p signal sometimes gave rise to the formation of a doublet. This was hypothesised to be due to a degradative event. In order to repress any protease activity the triple protease mutant *CST211* strain was used. Strain *CST211* contains point mutations to proteinases *PRC1, PRD1 and PEP4* (Parr et al., 2007, Garcia-Alvarez et al., 1987, Wolf and Fink, 1975). The co-sedimentation assay was performed with the *BY4742* and *CST211* strains in parallel and blotted for Ysy6p,

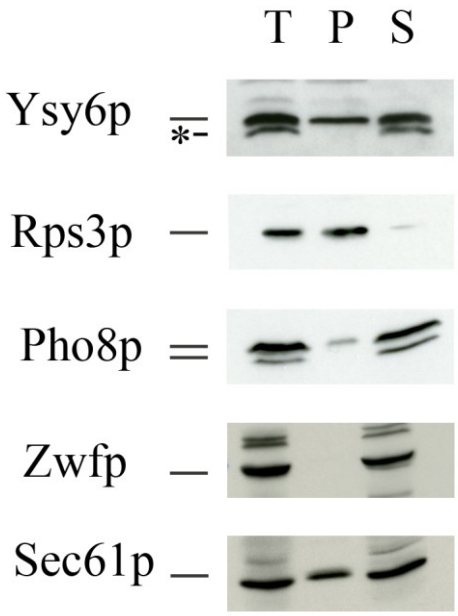


Figure 4.3 Further investigation of sedimentation of Ysy6p *BY4742 WT* and *Δysy6* strains were propagated in YPD media, lysed in HS buffer and solubilised in 3% (w/v) CHAPS, 10% (v/v) glycerol and sedimented through a high salt cushion. Total (T), Pellet (P) and supernatant (S) fractions were methanol precipitated prior to analysis by SDS-PAGE. 2 OD_{600} of cell culture equivalent were loaded onto a 15% SDS-PAGE gel for western blotting with Ysy6p antibody and 1 OD_{600} of cell culture equivalent was loaded onto 10% SDS-PAGE gels for blotting with Rps3p, Pho8p, Sec61p and Zwf1p antibody in 1xTBS 0.1% NP-40. Figure is representative of 2 repeat experiments.

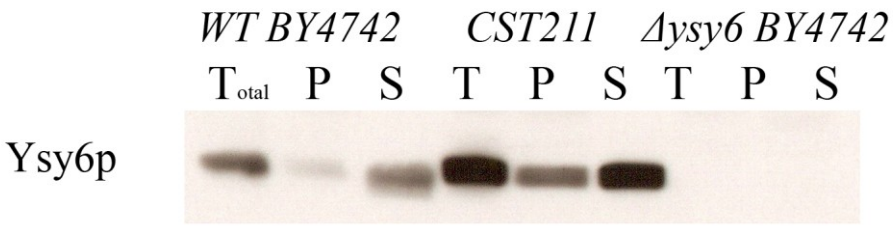


Figure 4.4 Effect of using a triple protease mutant strain on the cosedementation assay. *BY4742 WT*, Δ*ysy6* and *CST211* strains were propagated in YPD media, lysed in HS buffer and solubilised in 3% (w/v) chaps, 10% (v/v) glycerol and incubated at 30°C for 20 minutes prior to sedimentation through a HS cushion. Total (T), Pellet (P) and supernatant (S) fractions were methanol precipitated prior to analysis by SDS-PAGE and western blotting. 2 OD_{600} of cell culture equivalent were loaded onto a 15% SDS-PAGE gel for western blotting with Ysy6p antibody.

however the solubilised lysate was incubated at 30°C for 20 minutes prior to ribosome sedimentation. The temperature of 30°C was chosen as it the temperature at which yeast grows optimally and therefore hypothesised to be close to the optimum temperature for endogenous proteolytic enzymes (Watson, 1987). As shown in Figure 4.4 the sedimented fraction is of a slightly higher molecular weight than the fraction of Ysy6p present in the supernatant. In contrast, the triple protease mutant shows bands of equal molecular weight across all fractions.

Having implicated proteolytic degradataion in affecting cosedimentation of Ysy6p it was hypothesised that keeping the samples and reagents ice cold would abolish variations in sedimentation. To confirm this, the *BY4742* lysates were made and kept on ice or incubated at 30°C for 20 minutes. The samples were then centrifuged to sediment ribosomes. As shown in figure 4.5 heating the lysate nearly abolished sedimentation of Ysy6p and gave rise to the majority of Ysy6p remaining in the supernatant in a lower molecular weight form. However, keeping the lysate and reagents ice cold led to an increase in sedimentation of Ysy6p. It can be noted that ribosomes seem to have been less efficiently sedimented for the lysate kept ice cold. This suggests that the sedimented portion of Ysy6p may be underrepresented. It can be noted that even when the lysate is kept ice cold a marginal amount of degradation was still observed in the supernatant fraction. Ysy6p therefore cosediments with ribosomes in manner which is proteolytically sensitive.

4.3 Ysy6p comigrates with ribosomes

The cosedimentation assay showed that a pool of Ysy6p cosediments with ribosomes. However, cosedimentation could be caused by other factors such as aggregation or potential cosedimentation with other high molecular weight complexes. Therefore to more rigorously investigate the cosedimentation result polysomes were analysed by loading the P200 fraction onto a high salt 15-50% (w/v) sucrose gradient, centrifuged and fractionated from top to bottom followed by analysis by SDS-PAGE. In order to avoid loss of Ysy6p signal due to degradation, the triple protease mutant *CST211* strain was used.

As shown in figure 4.6, Rps3p, a component of the 40S subunit, and Rpl17p, a component of the 60S subunit, were detected in fractions 6 to 24. Both ribosomal

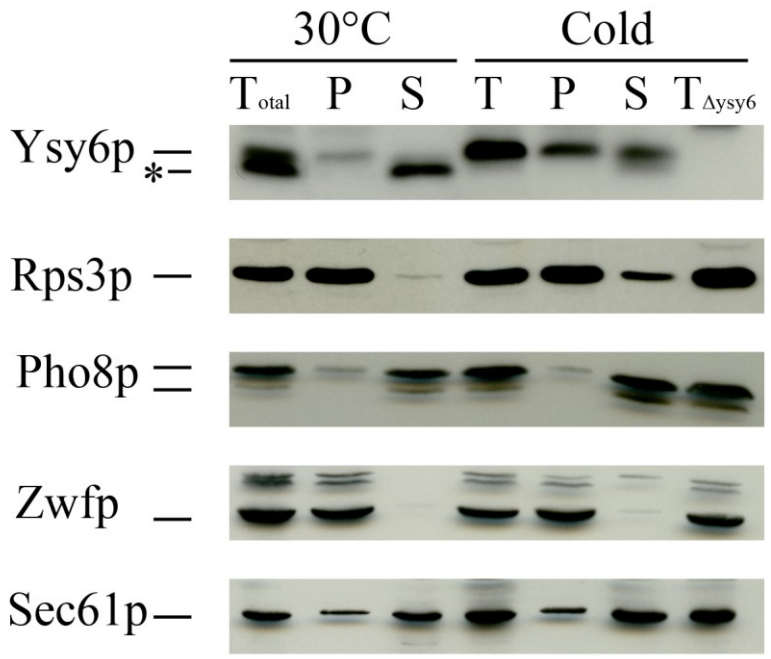


Figure 4.5 Effect of temperature on the cosedimentation of Ysy6p with ribosomes. *BY4742 WT* and Δ*ysy6* strains were propagated in YPD media, lysed in HS buffer and solubilised in 3% (w/v) CHAPS, 10% (v/v) glycerol and incubated on ice or at 30°C for 20 minutes prior to sedimentation through a high salt cushion. Total (T), Pellet (P) and supernatant (S) fractions were methanol precipitated prior to analysis by SDS-PAGE. 2 OD_{600} of cell culture equivalent were loaded onto a 15% SDS-PAGE gel for western blotting with Ysy6p antibody and 1 OD_{600} of cell culture equivalent was loaded onto a 10% SDS-PAGE gel for western blotting with Rps3p, Pho8, Sec61p and Zwf1p antibody.

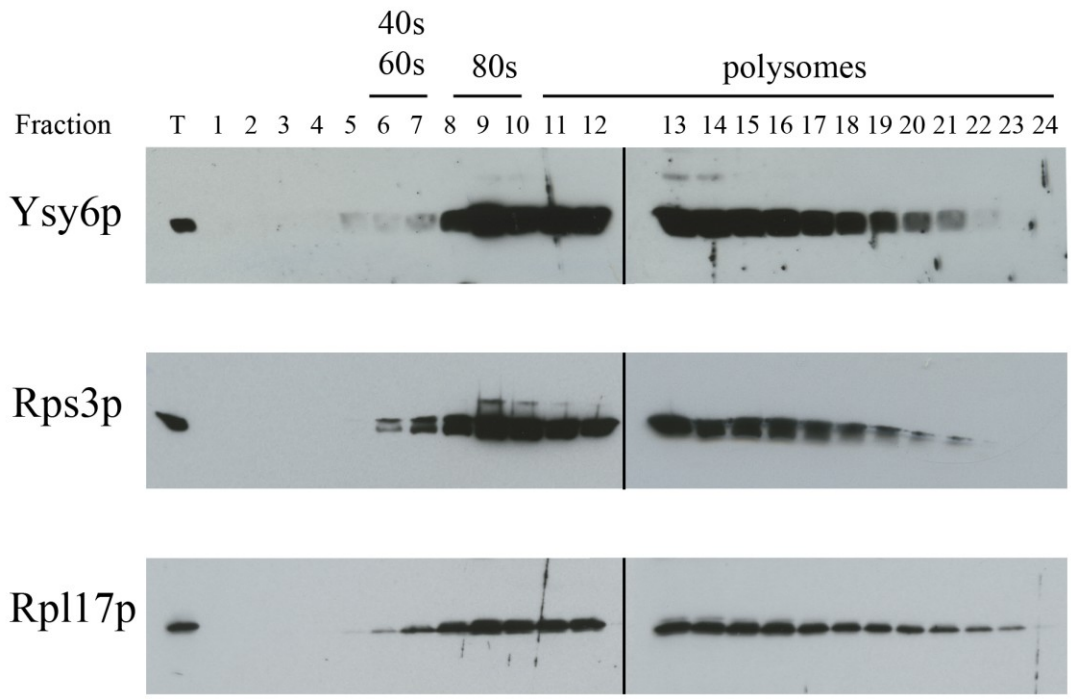


Figure 4.6 High salt sucrose gradient fractionation. Strain *CST211* was propagated in YPD media, lysed in HS buffer and solubilised in HS buffer with 3% (w/v) CHAPS, 10% (v/v) glycerol and sedimented through a high salt cushion. 600 OD_{600} units of cell culture equivalent of P fraction was then resuspended in 300 µl of HS Buffer 3% (w/v) CHAPS, 10% (v/v) glycerol and centrifuged at 16 000 x g for 20 minutes prior to loading onto an 11.5ml 15 to 50% (w/v) High salt sucrose gradient with 0.5% (w/v) CHAPS. Gradient was fractionated from top to bottom. Samples were analysed by SDS-PAGE and western blotting. For western blotting for Rpl17p and Ysy6p 100 µl of each fraction were methanol precipitated and loaded onto a 15% SDS-PAGE gel. For western blotting for Rps3p 20 µl of each fraction were directly loaded onto a 10% SDS-PAGE gel. The T fraction corresponds to the expected signal per lane assuming equal distribution of protein across samples.

signals increased from fractions 6 to 9 after which the signal decreased consistently. No ribosomal signal could be detected in fractions 1 to 5 indicating that ribosomes remained intact. Ysy6p could not be detected in fractions 1 to 4 and in trace levels in fractions 5 to 7. A sharp increase to a strong Ysy6p signal could be observed in fraction 8 which was increased to its maximum in fraction 9 followed by a gradual decrease until loss of signal in fraction 23. The Ysy6p and ribosomal signals therefore reached their maxima concomitantly at fraction 9. Therefore both the ribosomal and Ysy6p signals increase and decrease concomitantly and reach their maxima concomitantly. The Rps3p and Rpl17p signals however appear 2 to 3 fractions before the Ysy6p signal. This can be rationalised by the fact that free 40S and 60S subunits are present in the P200 fraction and migrate more slowly (Frey et al., 2001). The strongest signal in fraction 8 corresponds to fully assembled 80S followed by polysomes in higher fractions. The Ysy6p signal in fractions 5 to 7 could be due to either aggregation or non specific binding to ribosomes. The latter is less likely as the Ysy6p signal does not increase concomitantly with increase in Rpl17p across these fractions. However, Ysy6p shows a migration pattern through a high salt sucrose gradient which correlates to the migration pattern of fully assembled 80s ribosomes and polysomes.

4.4 Ysy6p cosedimentation is not saturated at endogenous levels

In order to obtain an insight into the binding kinetics of Ysy6p ribosome association, Ysy6p-opsin was used as an overexpression construct in the *WT BY4742* strain. It has been demonstrated that mammalian RAMP4 is recruited to ribosomes in a manner which is dependent on the emergence of a transmembrane domain in the exit tunnel (Pool, 2009). This suggests that recruitment of RAMP4 is highly regulated. It was hypothesised that if Ysy6p recruitment was also such a highly regulated process which is saturated at endogenous levels then cosedimentation of Ysy6p should not increase with over-expression.

As shown in figure 4.7, the signal from the Ysy6p antibody is of greater intensity when Ysy6p-opsin, under the control of the *MET25* promoter, is co-expressed with endogenous Ysy6p (opsin lane) than when endogenous Ysy6p is expressed alone (WT lane) in selective media lacking methionine and leucine. The P fraction demonstrates that, as for endogenous Ysy6p, Ysy6p-opsin also co-sediments with ribosomes. However, when Ysy6p is overexpressed the amount of co-sedimented material is

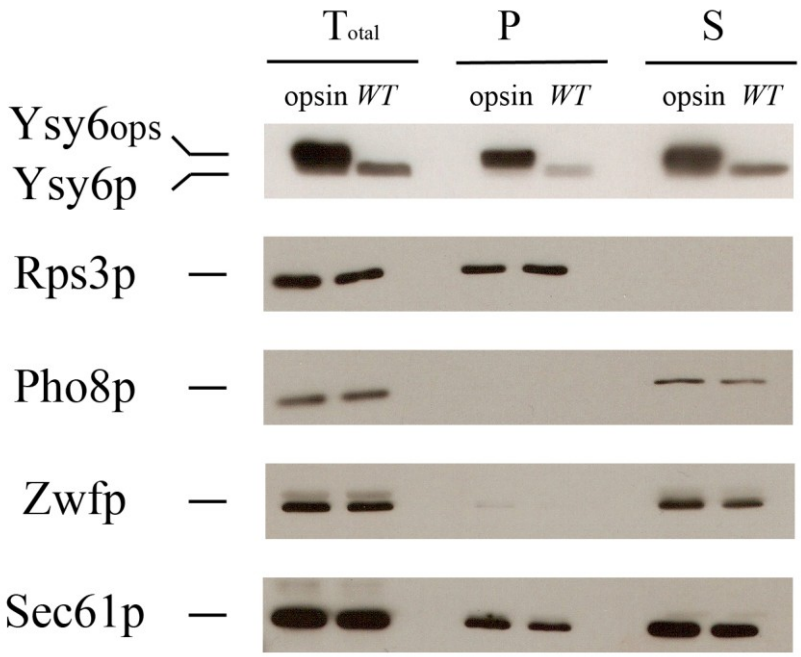


Figure 4.7 Comparative cosedimentation of Ysy6p and Ysy6p-opsin *BY4742 WT* and *WT* expressing Ysy6p-opsin strains were propagated in selective YNB media lacking leucine and methionine, lysed in HS buffer and solubilised in 3% (w/v) CHAPS, 10% (v/v) glycerol and sedimented through a high salt cushion. Total (T), Pellet (P) and supernatant (S) fractions were methanol precipitated prior to analysis by SDS-PAGE. 1 OD_{600} of cell culture equivalent were loaded onto a 15% SDS-PAGE gel for western blotting with Ysy6p antibody and 10% SDS-PAGE gels for western blotting with Rps3p, Pho8p, Sec61p and Zwf1p antibody. Representative of 2 repeat experiments.

increased relative to cells expressing endogenous Ysy6p alone in a manner which appears proportional to the total. Lastly, it can be seen that the amount of endogenous Ysy6p which cosediments with ribosomes when co-expressed with Ysy6p-opsin appears reduced relative to cells expressing endogenous Ysy6p alone. This suggests that Ysy6p and Ysy6p-opsin are competing for ribosome association and therefore that they have the same binding sites which mediate ribosome association.

No difference between the strains could be observed for experimental controls. Furthermore, as expected the Zwf1p signal was present exclusively in P fraction, the Pho8p and Zwf1p signals were present in the S fraction and the Sec61p signal present in both the P and the S fraction. The data therefore suggest that Ysy6p-opsin and Ysy6p have similar binding sites which mediate ribosome association. In addition, the data demonstrate that cosedimentation of Ysy6p is affected by its expression level and that cosedimentation of Ysy6p is not saturated at endogenous levels.

4.5 Investigating the effect of truncating Ysy6p-opsin

Having identified that Ysy6p and Ysy6p-opsin associate with ribosomes, the next step was to understand which sequences were required for ribosome binding. In order to investigate this sequence alignment of Ysy6p and its mammalian homologs RAMP4-1 and RAMP4-2 were performed and identified two regions of conservation (Figure 4.8 (a)). For Ysy6p this corresponds to residues 7 to 18 and 44 to 64. Hydropathy profiling by the methodology from Hofman and Stoffel (1993) maps the transmembrane segment of Ysy6p from residues 43 to 59 (Figure 4.8 (b)). Ysy6p therefore contains a conserved cytosolic and transmembrane domain.

Yamaguchi et al., (1999) demonstrated that RAMP4 can be crosslinked to the Sec61 complex and Gorlich and Rapaport (1993) demonstrated that a small proportion of RAMP4 stays associated with the Sec61 complex after removal of ribosomes. Together this suggests that the transmembrane domain of RAMP4 is tightly associated with ribosomes via the Sec61p complex. However, Gorlich and Rapaport (1993) also observed that removal of ribosomes greatly reduces the amount of RAMP4 which is associated with Sec61. Furthermore, Pool (2009) demonstrated that RAMP4 can be crosslinked to rpL17 which is suggestive of association of cytosolic domains of

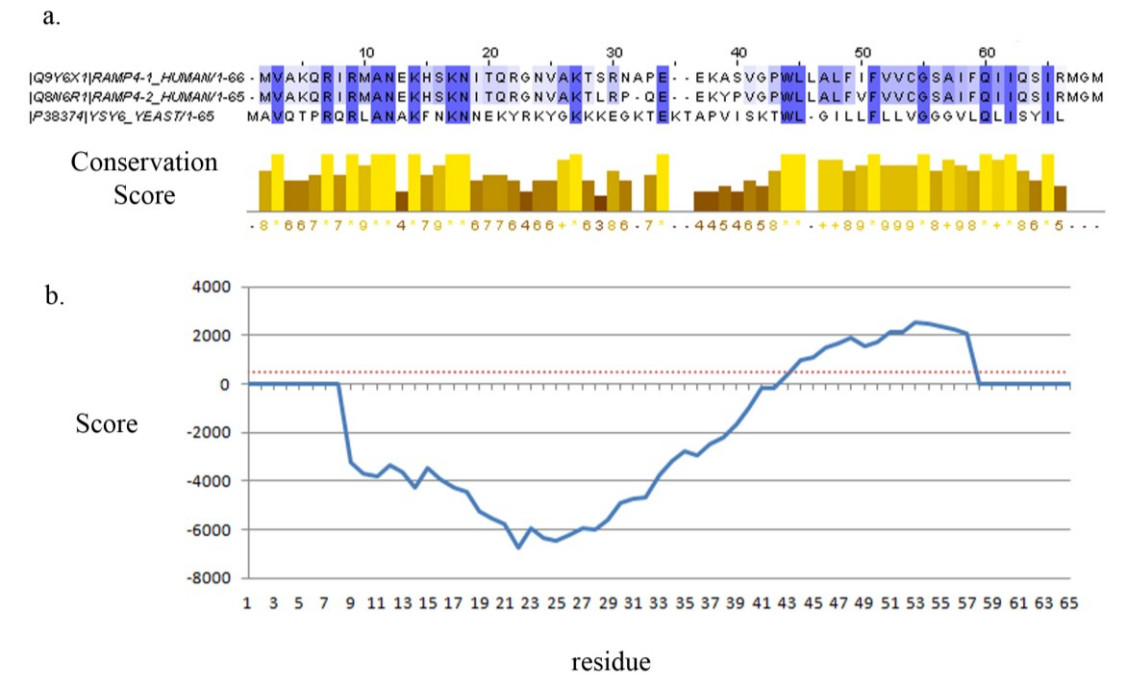


Figure 4.8 Bioinformatic analysis of Ysy6p. (a.) Sequence alignment using Clustal Omega of Ysy6p from *S. cerevisiae* and human Ramp4-1 and Ramp4-2 from uniprot referenced sequences coloured according to sequence conservation (Sievers et al., 2011). (b.) Transmembrane domain prediction according to Hofmann and Stoffel (1993) positive values with values of over 500 (red dotted line) for segment maxima are predicted to be transmembrane domains.

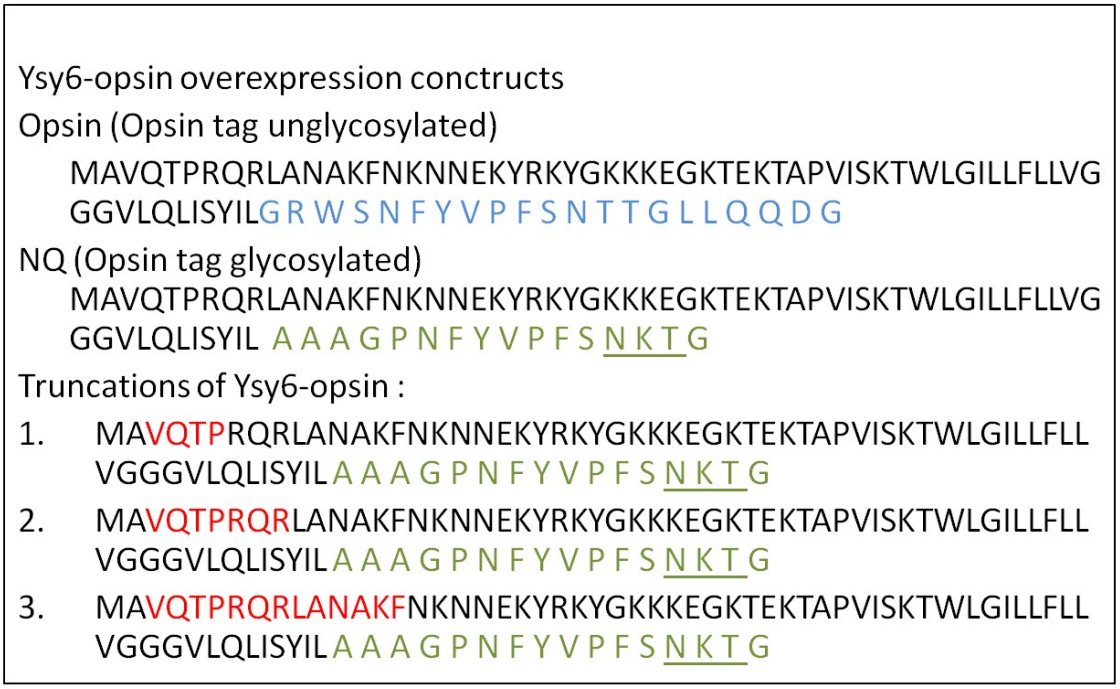


Figure 4.9 Sequence of Ysy6p-opsin and its truncations. The sequence of the Ysy6p-opsin constructs were confirmed using internal forward and reverse primers for *YSY6*. Two opsin tagged Ysy6 constructs were used. The Unglycoslyatable opsin tag (blue), Glycosylatable opsin tag (green) with the glycosylation site NKT (underlined green) (Gavel and von Heijne, 1990). Three truncations of Ysy6 opsin were made T1, T2 and T3 which correspond to sequenctially bigger deletions of the *YSY6* open reading frame (red).

RAMP4 with the ribosome. Therefore ribosome association of RAMP4 could be mediated via association of its transmembrane domain with the Sec61p complex or via cytosolic domains with the ribosome. Considering that Ysy6p and RAMP4 have a conserved cytosolic region and transmembrane domain it was hypothesised that these domains could promote ribosome association. Therefore, to test whether the conserved cytosolic domain was required for ribosome binding, sequence deletions of Ysy6p-opsin were made and expressed in the *Δysy6 BY4742* background. The tagged Ysy6p construct was chosen as the Ysy6p antibody was raised to the N terminus and may not have recognised the truncated proteins.

The truncations made are shown in figure 4.9. The N terminal Met residue was not deleted as it is required for transcription and translation initiation and is encoded by the kozak consensus sequence (Kozak, 1987). In addition, the P2 residue was not removed due to concerns that protein integration into the ER may be affected (Forte et al., 2011). Truncation 1 (T1) corresponds to deletion of resiudes 3 to 5. T2 corresponds to deletion of residues 3 to 8. Finally, T3 corresponds to deletion of residues 3 to 14.

Truncations were made using phosphorylated primers which amplify the Ysy6p-opsin encoding vector excluding the sequences for the required deletions. The resulting constructs were verified by sequencing using forward and reverse primers for Ysy6p which overlap for the area sequenced. This demonstrated that the truncations contained no mutations in the Ysy6p open reading frames and that the deleted areas caused no shift in reading frame (Appendix II).

Having verified the vector DNA sequences expression profiles of the proteins was verified separation on 20% SDS-PAGE gels and western blotting. As can be seen in figure 4.10 the truncations run faster than the untruncated construct. T1 migrates marginally faster than the untruncated protein. Truncations T2 and T3 migrate faster in correlation with extent of truncation. The T2 and T3 constructs do not show any glycosylated form of the protein. As shown by the untruncated protein, glycosylation is only poorly efficient. Short exposures revealed that there is in fact less of the T2 and T3 truncations present. However, this was not due to unequal protein content of the sample as Sec61p levels remained similar across samples. The absence of the glycosylated forms of T2 and T3 may therefore be caused by inefficient glycosylation or decreased load due to either a decrease in truncated protein concentration in the sample or

inefficient transfer during western blotting. As shown in figure 4.10 the first truncation cannot be recognised by the Ysy6p antibody thus demonstrating the requirement of using the tagged overexpresion construct.

4.5.1 Truncating Ysy6p-opsin causes loss of ribosome association

The cosedimentation assay was performed with cells expressing the Ysy6p-opsin constructs. A fraction of the full length glycosylatable construct was sedimented. In addition truncation T1 could also be sedimented in a similar proportion (Figure 4.11). However, only trace amounts of truncations T2 and T3 could be sedimented. The combined P and S fractions appear to be greater than the T fraction. This is due to the fact that the gel is overexposed. The gel was overexposed to over-represent the P fraction. This was done out of concern that due to the lower expression levels of T2 and T3 the signal from the P fraction for these truncations was simply weaker in relation to the total. However, T2 and T3 total levels do not appear dissimilar to untruncated Ysy6p-opsin and truncation T1. For these reasons the loss of sedimentation in truncations T2 and T3 cannot be due to a lack of detection due to reduced protein yield in lysates from truncation T2 and T3 expressing cells. Sec61p demonstrated similar levels of sedimentation across all samples which is indicative that there was no variation in efficiency in cosedimentation of ribosome associated proteins across samples. The Zwf1p blot shows that there was no cytosolic contamination of the P fraction. The Rps3p signal shows that not all, but the majority of the ribosomal material has been sedimented and that the proportion of unsedimented material is consistent across samples. Pho8p shows trace amounts of sedimentation in the truncated fractions. This may be indicative of non specific binding to ribosomes or aggregation. In support of the latter theory is the fact that Truncation T2 shows weaker sedimentation than T3. Taken together the data suggests that Truncation T2 does not associate with ribosomes while truncation T1 is ribosome associated to a similar degree as untruncated Ysy6p-opsin. This shows that the poorly conserved residues 3 to 6 are not required for ribosome association and that loss of residues 7 to 9 which constitute only part of the highly conserved cytosolic sequence is sufficient to cause loss of ribosome association.

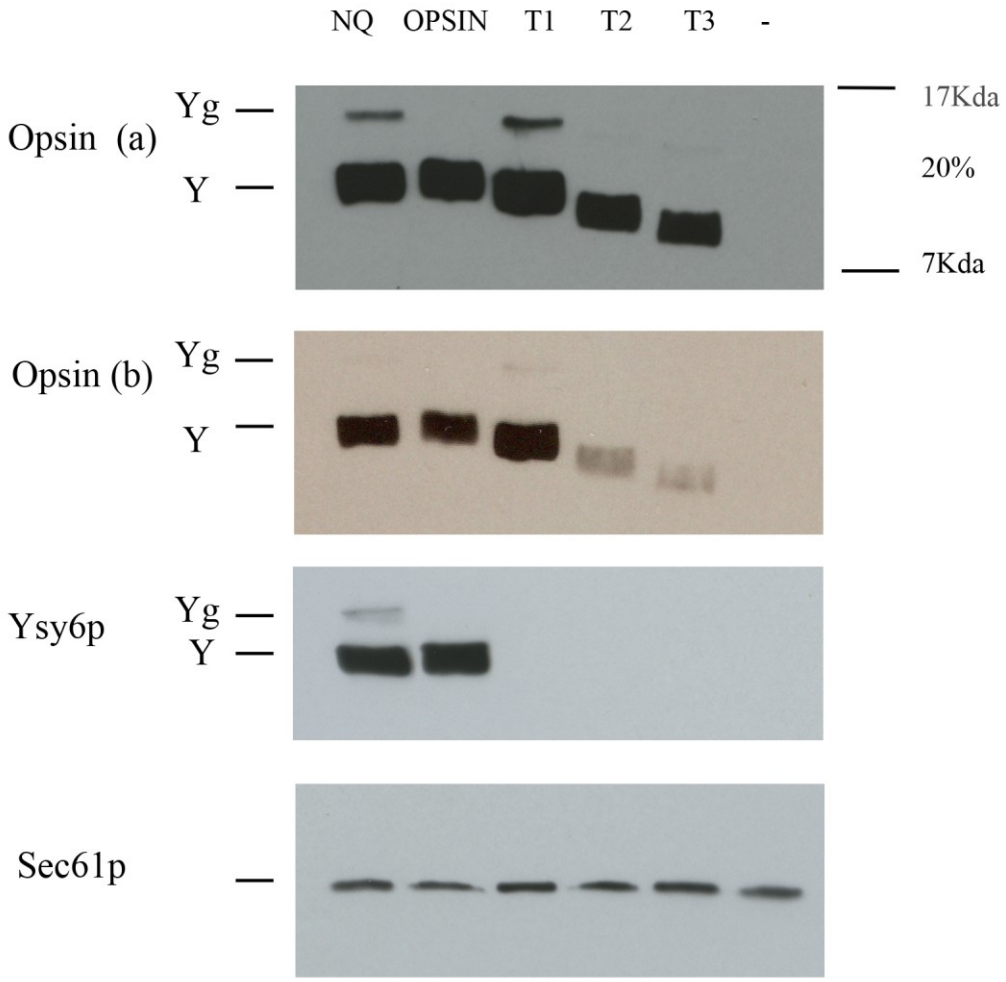


Figure 4.10 Expression of Ysy6p-opsin constructs. P16 membrane preparations were prepared in LS buffer. 2 OD_{600} of cell culture equivalent of lysates from strain *BY4742 Δysy6* transformed with pRS315 or plasmids encoding for truncations T1, T2, T3, glycosylatable untruncated Ysy6p-opsin (NQ) or untruncated unglycosylatable Ysy6p-opsin (opsin) constructs were loaded onto SDS-PAGE gels. 20% SDS-PAGE gel was used for western blotting with anti opsin anitbody, 15% SDS-PAGE gel for western blotting with anti Ysy6p antibody and 10% SDS- PAGE gel for blotting with Sec61p. Two exposures of the same gel are shown for the anti opsin blot (a and b). Glycosylated Ysy6p-opsin and deletion constructs thereof are denoted as Yg and unglycosylated as Y.

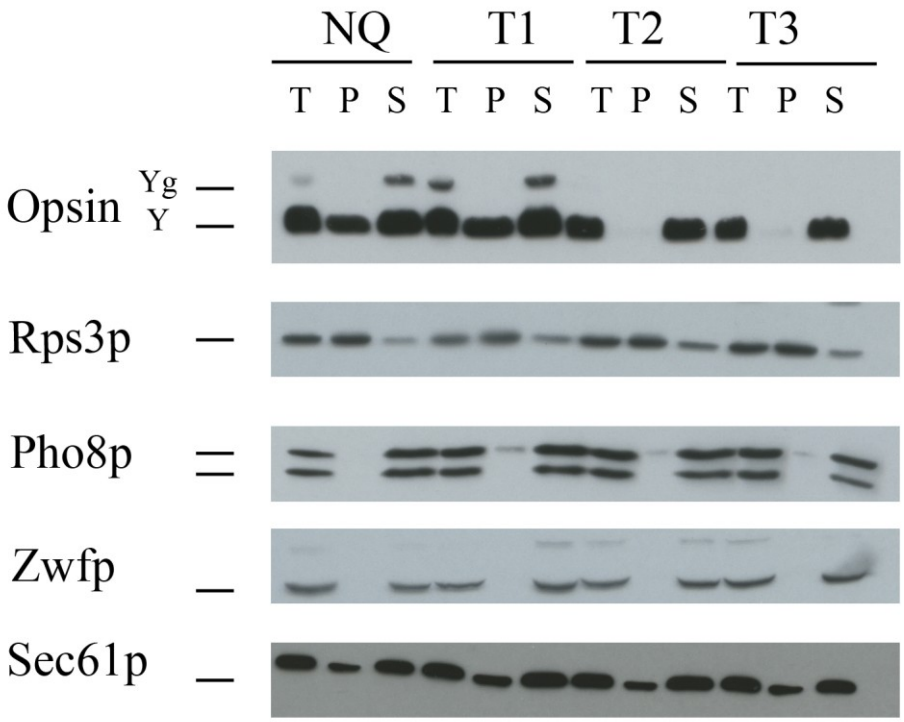


Figure 4.11 Investigation of cosedimentation of truncated products. The *BY4742 Δysy6* strain expressing truncations T1, T2, T3 or untruncated glycosylatable Ysy6p-opsin (NQ) were propagated in minimal SD media lacking leucine and methionine, lysed in HS buffer and solubilised in HS buffer with 3% (w/v) CHAPS, 10% (v/v) glycerol and sedimented through a high salt cushion. Total (T), Pellet (P) and supernatant (S) fractions were methanol precipitated prior to analysis by SDS-PAGE and western blotting. 2 OD_{600} of cell culture equivalent were loaded onto a 15% SDS-PAGE gel for western blotting with Ysy6p antibody and 1 OD_{600} of cell culture equivalent was loaded onto a 10% SDS-PAGE gel for western blotting with Rps3p, Pho8, Sec61p and Zwf1p antibody. Western Blotting for Ysy6p representative of 3 repeat experiments. Glycosylated constructs are denoted as Yg and unglycosylated as Y.

4.5.2. Loss of ribosome association cannot be attributed to loss of protein integration at the ER

Truncations T2 and T3 fail to co-sediment with ribosomal material. However, this may not be due to loss of capacity to associate with ribosomes but may be the result of defective protein integration at the ER due to lack of recognition by the GET complex (Schuldiner et al., 2008). In 4.5.1 it was shown that a conserved cytosolic region is required for ribosome cosedimentation. However, there are two distinct models that can be put forward for ribosome association of Ysy6p. Firstly, Ysy6p associates directly with the ribosome via its cytosolic domain. Secondly, it associates indirectly with the ribosome via its cytosolic domain by binding to other factors which are tightly bound to the ribosome. In the latter scenario, it is most probable that correct protein integration of Ysy6p would be required as incorporation into this complex would be a prerequisite for ribosome association. Therefore, the topology of the Ysy6p-opsin constructs were investigated.

4.5.2.1 Ysy6p-opsin truncations are membrane associated

In order to investigate protein integration of Ysy6p-opsin and its truncations it was first investigated whether the constructs were soluble or membrane associated. This was done by making crude membrane preparations which cosediment at between 1 200g and 16 000g. The membrane preparation includes the majority of ER associated material as well as nuclear and mitochondrial material whereas the supernatant contains soluble components (Frey et al., 2001). The total lysate (T) was then compared to the sedimented material (P) and the soluble material (S). This was done by analying the T, P and S fractions using SDS-PAGE gels and western blotting using the anti opsin, Sec61p and Zwf1p antibodies (Figure 4.12). Ysy6p-opsin protein constructs all cofractionated with membranes as T and P fractions gave rise to opsin signals of comparable intensity with no opsin signal from the S fraction. The Sec61p signal demonstrated a similar profile to the opsin signal with all of Sec61p fractionating into the P fraction. This demonstrates that ER was efficiently sedimented. The cytosolic control Zwf1p signal was present in the S fraction at a comparable level to the T fraction. Only trace amounts of Zwf1p could be detected in the P fractions of unglycosylatable Ysy6p-opsin and truncations T1 and T2. The Ysy6p-opsin constructs are therefore membrane associated.

4.5.2.2 Proteinase K digestion of Ysy6p-opsin

As shown previously, the Ysy6p-opsin constructs are membrane associated. However, in order to demonstrate that Ysy6p-opsin was integrated into the ER in the correct topology, a proteinase protection assay using Proteinase K was carried out. As shown in Figure 4.13 Ysy6p-opsin may exist in three different topologies: a cytosolic N terminus, cytosolic C terminus or peripherally associated to the ER with cytosolic N and C termini. Depending on the topology, the N and C terminus will be differentially sensitive to protease digestion. In the event of the Ysy6p-ospin construct being peripherally associated the N and C termini will both be sensitive to protease digestion. In the event of a cytosolic N terminus the C terminal opsin tag will be protected from digestion. Finally, in the event of a lumenal N terminus the opsin tag will be sensitive to Proteinase K. Kar2p is a lumenal protein which was used as a marker for membrane integrity (Normington et al., 1989). When membrane preparations are made no digestion of Kar2p should occur however after solubilisation of the ER with 0.5% (v/v) Triton X-100 Kar2p will no longer be protected from digestion.

Proteinase K digestion was carried out using P16 membrane preparations from the *BY4742* Δ*ysy6* strain expressing the Ysy6p-opsin constructs. As shown in figure 4.14 (a and b) the full length opsin constructs were blotted for the N terminus using Ysy6p antibody and the C terminus using anti opsin antibody. For both full length Ysy6-opsin constructs the Ysy6p antibody signal progressively decreased with increasing concentrations of Proteinase K. Strikingly, no intermediate digestion products could be detected suggesting that Ysy6p-opsin is present in the correct topology with a cytosolic N terminus. When digestion was carried out in the presence of detergent the Ysy6p antibody signal (-1 and -2) became weaker at lower concentrations suggesting that Triton X-100 enhances digestion of Ysy6p-opsin. The opsin signal showed a shift in size of the order of 4 kDa when digestion was carried out without the presence of Triton X-100. The lower band (*1) intensity increased with concentration of Proteinase K whereas the higher band (*2) decreased to trace levels at the highest concentration. When digestion was carried out in the presence of detergent there was disappearance of the higher band but no accumulation of lower molecular weight product. Instead there was a gradual disappearance of opsin signal combined with gradual decrease in the size. This demonstrates that the opsin antibody detects the presence of a protected C terminal fragment of Ysy6p-opsin in the absence of Triton X-100 which is consistent with the

topology of a tail anchored protein. Kar2p is an 80kDa ER lumenal protein which is therefore protected in the absence of detergent (<1) but is sensitive to digestion in the presence of Triton (<2) (Normington et al., 1989). Protection of Kar2p therefore showed that the membrane preparations consisted of membranes which were intact and not permeable to Proteinase K. Loss or change in size of the signals from both the Ysy6p and opsin antibodies were therefore caused by digestion of cytosolic components. The digestion profile is consistent with digestion of tail anchored protein with sensitive N and protected C terminus. Schuldiner et al., (2008) have already demonstrated that the Ysy6p-opsin construct is integrated at the ER in the correct topology due to the fact that Ysy6p-opsin can be glycosylated. However, the efficiency of this glycosylation is poor which can be rationalised in two ways. Firstly, only a small proportion of Ysy6p-opsin is integrated at the ER or glycosylation of this site is inefficient. However, these results show that both the glycosylated and unglycosylated Ysy6p-opsin show a similar degree of sensitivity to Proteinase K at their N terminus (-1 and -2). This suggests that Ysy6p-opsin is inefficiently glycosylated but efficiently integrated.

The truncations of Ysy6p-opsin gave rise to similar digestion profiles to the untruncated constructs. Across all truncations the anti opsin antibody signal gave rise to an accumulation of a protected fragment (*2) with increasing concentrations of Proteinase K and which was inversely correlated with the undigested protein signal (*1). In the presence of Triton X-100 the protected fragment did not appear and the opsin antibody signal gradually decreased. However, digestion in the presence of Triton X-100 gave rise to concentration specific fragments. These fragments disappeared with increased concentrations of Proteinase K and therefore constitute concentration specific digestion intermediates. In addition, these fragments were detectable as a smear in the untruncated constructs. Kar2p was protected from digestion in the absence of Triton X-100 (<1) in truncations T2 and T3. Kar2p was also protected in truncation T1 in the absence of Triton X-100. However, Kar2p fragments of low intensity could be detected in truncation T1 in the absence of Triton X-100 but this did not ostensibly impact on the level of full length protein and therefore does not impact on the interpretation of the results. The appearance of this band suggests that the integrity of a minor proportion of the membrane preparations used was compromised. This is most probably caused by shearing during the process of glass bead cell lysis. Importantly, in the presence of

Triton X-100 Kar2p was completely digested (<1 and <2). However, subtle variations exist in the rate of disappearance of full length and digested Kar2p (<1 and <2) however these variations are not specific to membranes prepared from cells expressing the truncated products as membranes from cells expressing the untruncated products also displayed similar variations.

All constructs showed accumulation of a protected fragment when blotted with anti opsin antibody which disappeared in the presence of Triton X-100. The truncations, however, appeared to be refractory to digestion in comparison to the full length constructs as at 1mg/ml in the absence of Triton X-100 full length Ysy6p-opsin is nearly completely degraded (*1) however the truncations still show a substantial proportion of undegraded protein at 1mg/ml (*1). Two explanations for this are: firstly, truncating Ysy6p-opin renders the protein refractory to degradation or; secondly, there was an element of mixed topology. However, at least 50% of T1, T2, T3 must be in the correct topology as could be seen by the relative ratio of the opsin signal at 1mg/ml in the absence of detergent. In addition, this change in digestion profile did not correlate with a change in ribosome sedimentation. In summary it has been demonstrated that the Ysy6p-opsin constructs show a proteinase protection profile consistent with the topology of a tail anchored protein.

4.6 Discussion

It has been demonstrated that a fraction of Ysy6p cosediments with ribosomes and comigrates with ribosomes in sucrose gradients in a salt and detergent resistant manner. In addition, Ysy6p was shown to co-migrate in sucrose gradients with fully formed 80s but not 40s or 60s ribosomal subunits following membrane solubilisation, which further suggests that ribosome association of Ysy6p is not due to nonspecific binding to ribosomal RNA or protein and that Ysy6p associates selectively with fully formed ribosomes. This, therefore, demonstrates that similarly to the mammalian homolog RAMP4, a fraction of Ysy6p is in tight association with ribosomes and therefore that Ysy6p is a RAMP (Görlich and Rapoport, 1993).

Work by Schroder et al., (1999) and Pool (2009) demonstrated that recruitment of RAMP4 to ribosomes is dependent on co-translational integration of proteins in a

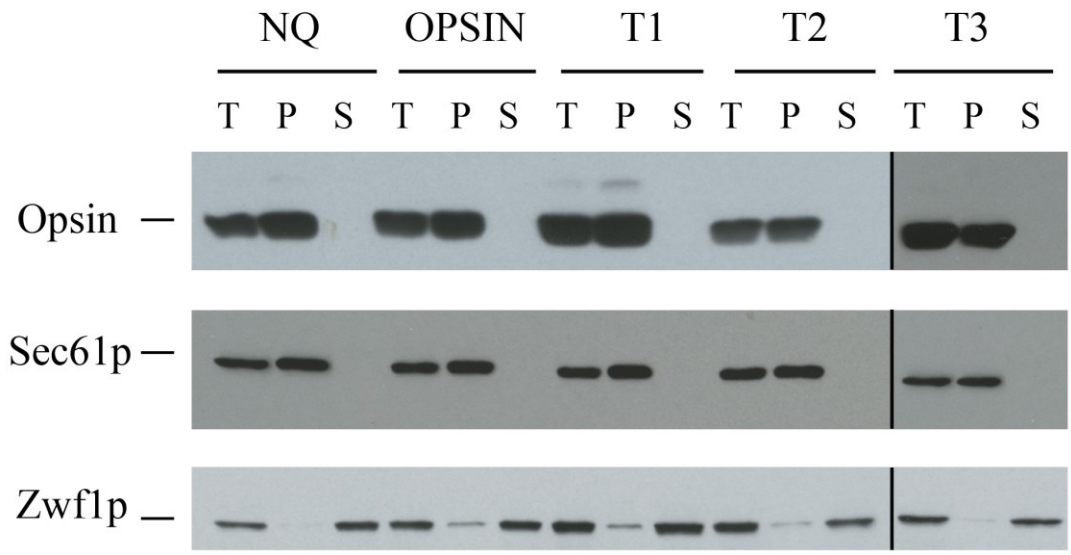


Figure 4.12 Investigating whether Ysy6p constructs are membrane associated. Total lysate was made by glass bead lysis in low salt buffer and the supernatant obtained after centrifugation at 1 200 x g was taken as the total lysate. The total lysate (T) was centrifuged at 16 000 x g for 20 minutes to obtain the P (pellet) and S (supernatant) fractions. 0.6 OD_{600} of cell culture equivalent of strain *BY4742 Δysy6* expressing truncations T1, T2, T3 or glycosylatable untruncated Ysy6p-opsin (NQ) constructs were loaded onto SDS-PAGE gels of 15% for western blotting with opsin antibody and 10% for western blotting with Sec61p and Zwf1p antibodies. Results from two SDS-PAGE gels separated by black line.

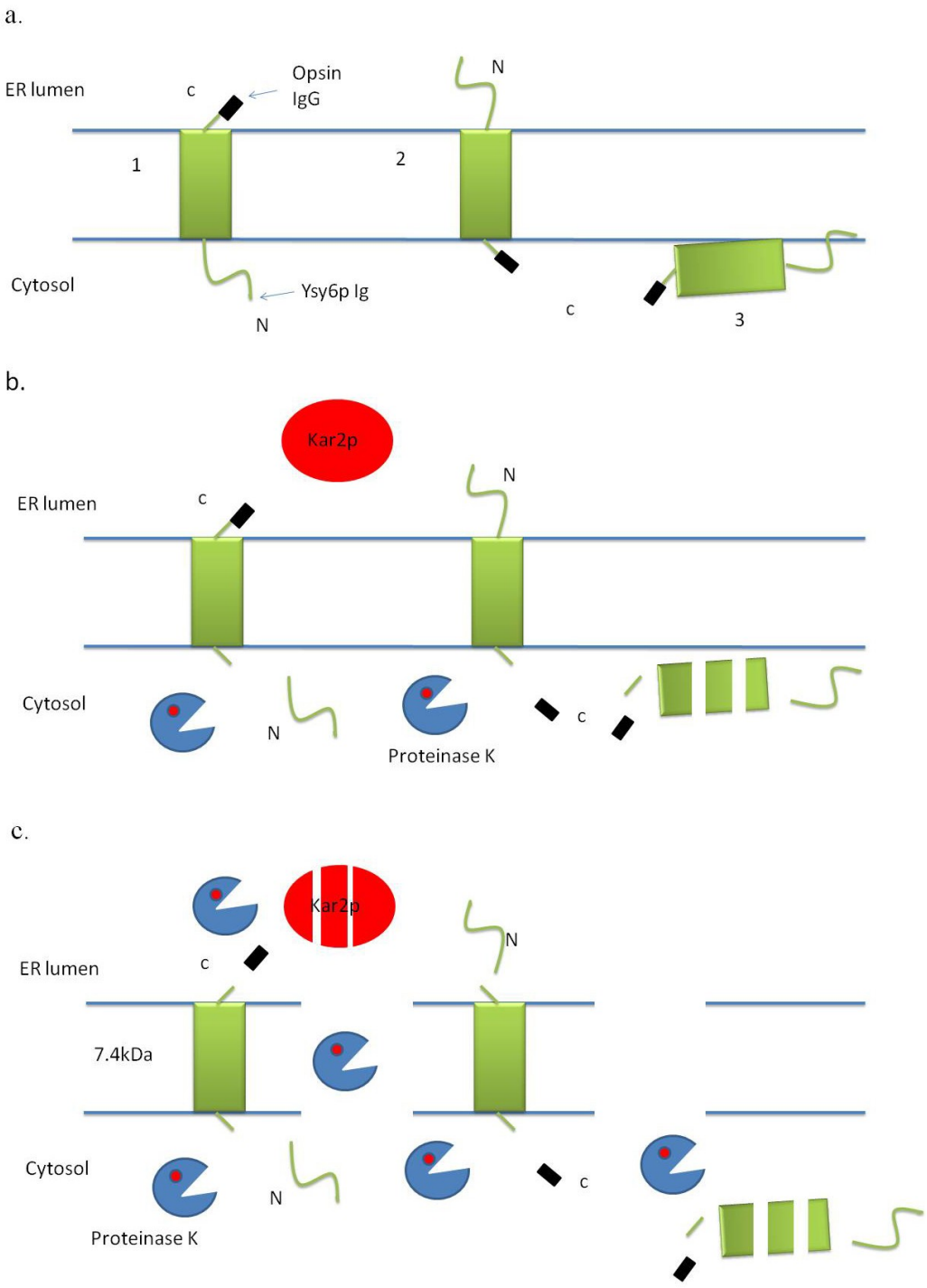


Figure 4.13 Overview of the Proteinase K protection assay. (a) The Ysy6p-opsin constructs can be associated with membranes in three different topologies (1, 2 and 3). The constructs contain a C terminal opsin tag (black) and the untruncated constructs are also recognised at their N terminus by the Ysy6p antibody (Ysy6 Ig) (b.) Proteinase K digests cytosolically exposed components of membrane associated proteins. Lumenal proteins are protected by the ER membrane. Depending on the orientation of the Ysy6p construct the opsin construct will shows sensitivity or protection to the Ysy6p and opsin antibodies (c.) Upon disruption of membrane integrity with Triton X-100 lumenal components become available for digestion with Proteinase K.

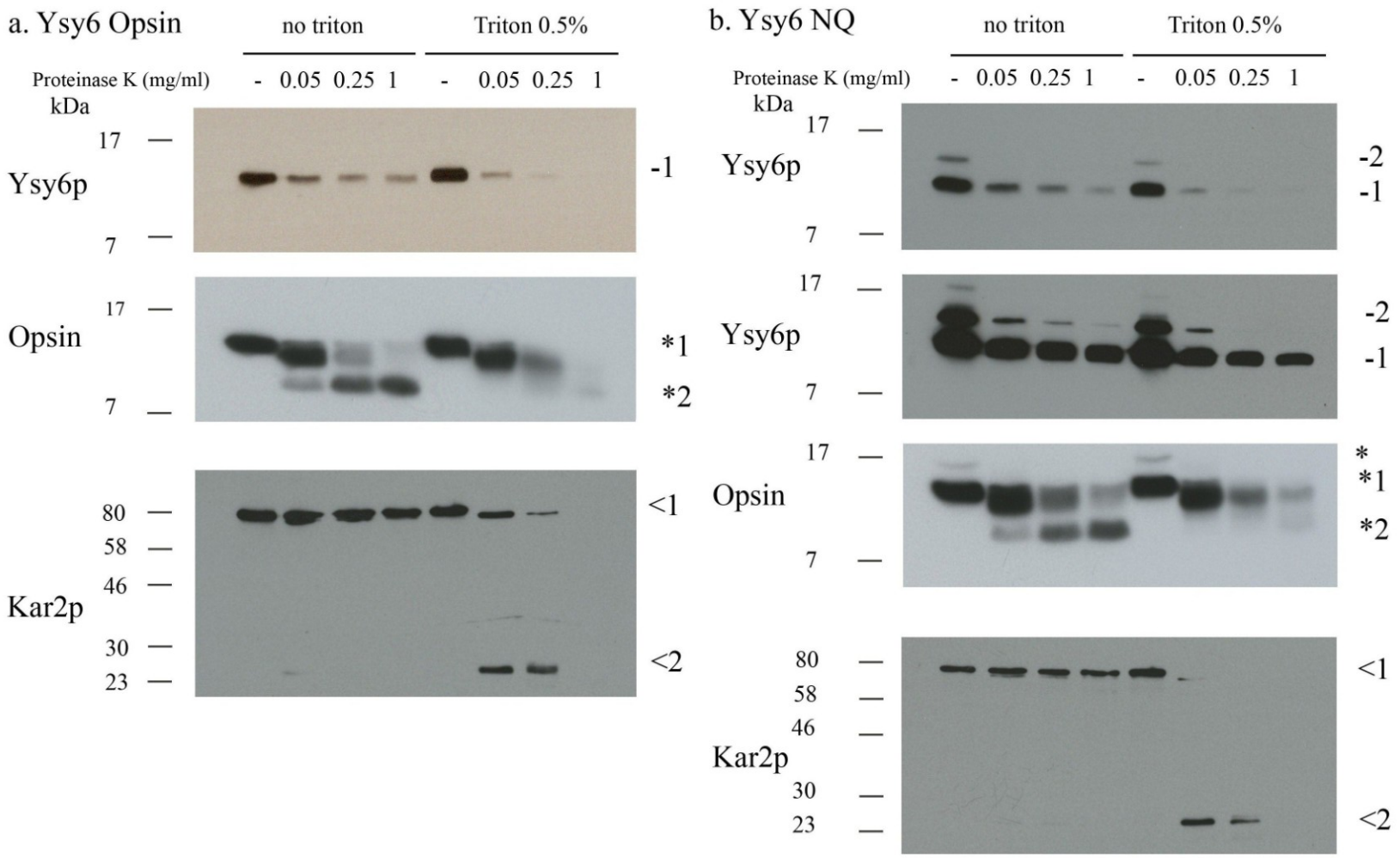


Figure 4.14 Digestion of P16 membrane preparations with Proteinase K. See page 118 for legend

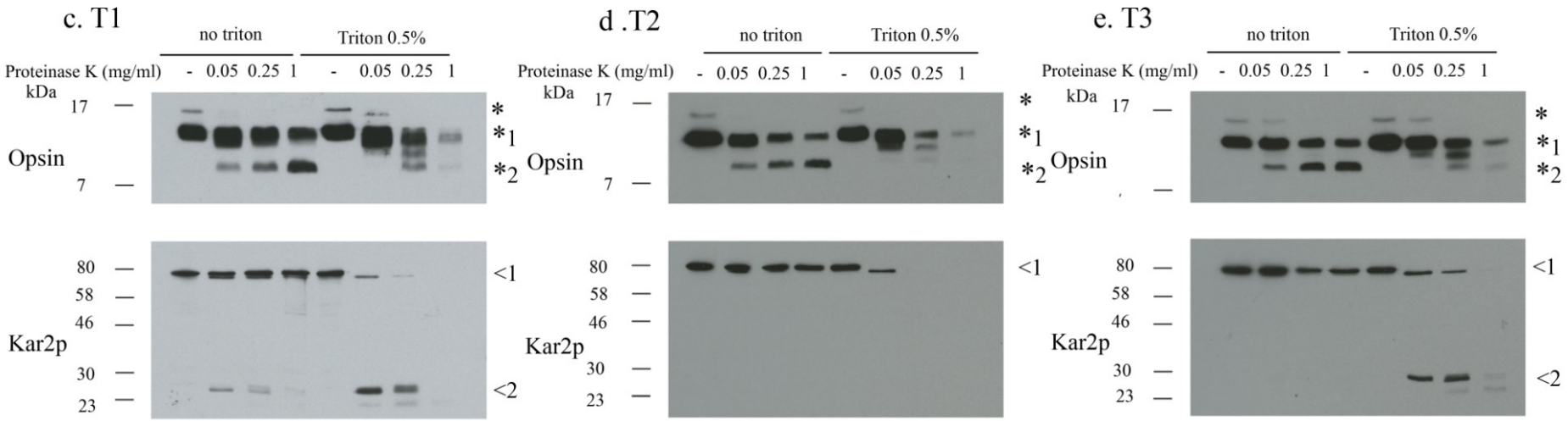


Figure 4.14 Digestion of P16 membrane preparations with Proteinase K. Crude P16 membrane preparations were made and treated with the indicated concentrations of Proteinase K (mg/ml) in the presence or absence of 0.5% (v/v) Triton X-100 (no triton or Triton 0.5%). Samples were analysed by SDS-PAGE and western blotting. 2 OD_{600} units of cell culture equivalent of sample was loaded onto 15% SDS-PAGE gels for western blotting with anti opsin antibody and anti Ysy6p antibody and 10% SDS-PAGE gels for western blotting with anti Kar2p antibody. Several bands representing the Ysy6p-opsin constructs are shown which include: the undigested, glycosylated protein (-2 and *); the undigested, unglycosylated protein (*1 and -1); and a partially digested protein fragment (*2). In addition, bands representing Kar2p are indicated for fulllength Kar2p (<1) and partially digested Kar2p (<2). Experiments were carried out using membrane preparations containing: (a.) non-glycosylatable Ysy6p-opsin, (b.) glycosylatable Ysy6p-opsin, (c.) Truncation T1, (d.) Truncation T2 and (e.) Truncation T3.

manner which is dependent on transmembrane domains of the nascent protein. This suggests that recruitment to ribosomes of RAMP4 is a regulated process. It was hypothesised that if Ysy6p was recruited to ribosomes as part of a preassembled stoichiometric complex then co-sedimentation of Ysy6p with ribosomes should not increase with over-expression. Consistent with this model is work by Wang and Dobberstein (1999) which showed that RAMP4 exists in a complex which does not contain the Sec61p complex or ribosomes. Alternatively, if Ysy6p is recruited on its own in a highly regulated manner then recruitment of Ysy6p should be limited by factors triggering recruitment of Ysy6p (i.e. hydrophobic segments of the nascent chain). In addition, considering that only a fraction of Ysy6p cosediments with ribosomes, unless the unassociated fraction of Ysy6p is sequestered by other proteins, this would mean that the majority of Ysy6p is available for ribosome association and therefore that endogenous Ysy6p levels are not limiting. To obtain an insight into the binding kinetics the effect of over-expression of Ysy6p-opsin on cosedimentation was investigated. It was demonstrated that cosedimentation of Ysy6p could be increased by over-expression thereby demonstrating that ribosome association is not saturated at endogenous levels. The implication of this is firstly, that Ysy6p is not recruited to ribosomes as part of a strictly stoichiometric complex. Secondly, that Ysy6p cosedimentation is dependent on expression level and therefore that the recruitment process of Ysy6p to ribosomes is not the limiting factor in cosedimentation at endogenous levels.

In order to investigate a potential role of the cytosolic domain of Ysy6p in ribosome association, truncations of the Ysy6p-opsin over-expression construct were carried out corresponding to deletions of segments of the cytosolic domain of Ysy6p-opsin. This showed that deletion of the sequence VQPT (truncation T1) did not affect sedimentation of Ysy6p-opsin. However, additional deletion of the sequence RQR (truncation T2) caused a near loss of ribosome association of Ysy6p-opsin. In addition, Proteinase K digestion of the Ysy6p constructs showed that loss of ribosome association was not due to defective integration into the ER. This demonstrates that the cytosolic domain promotes ribosome association of Ysy6p-opsin.

Sequence comparison demonstrated conservation of cytosolic and transmembrane domains across human homologs of RAMP4 and Ysy6p. In order to obtain a more global picture of sequence conservation of Ysy6p a more extensive protein alignment

of Ysy6p was performed as shown in figure 4.15. The sequence of Ysy6p is less conserved across organisms than previously observed from comparison of Ysy6p and its human homologs. However, the region corresponding to the transmembrane domain of Ysy6p is still conserved (amino acids 43 to 65) and the previously observed conserved cytosolic domain (amino acids 7 to 18) is shown to be part of greater more moderately conserved sequence (amino acids 1 to 29) which contains 2 strictly conserved residues. Strikingly, cytosolic amino acids 30 to 40 remain extremely poorly conserved.

In order to obtain an insight into the potential structure of the cytosolic domain of Ysy6p, the cytosolic domain was modelled using Quark (Xu and Zhang, 2012). As shown in figures 4.14 and 4.16 this gave rise to a helix-turn-helix motif for amino acids which are conserved across organisms. Strikingly, truncation T2 relates to deletion of residues located near the N terminus of the first helix. It was demonstrated that deletion of RQR is sufficient to cause near loss of ribosome association. However, due to the fact that RQR sequence is part of a greater conserved helix-turn-helix motif it is unlikely that the sequence RQR is sufficient on its own to promote ribosome association. Notably, the two strictly conserved residues are located in the predicted turn region. Taken together the data suggest that Ysy6p is composed of two distinct domains: a conserved helix-turn-helix domain which promotes ribosome association and a transmembrane domain which are linked by a poorly conserved linker peptide. However, it can't be excluded that the transmembrane domain also promotes ribosome association as cytosolic deletions do not cause a complete loss of cosedimentation of Ysy6p-opsin. This is consistent with observations by Görlich and Rapoport (1993) that 4% of cosedimented RAMP4 co-elutes with the Sec61 complex after removal of ribosomes and therefore suggests that the transmembrane and cytosolic domains of Ysy6p may act synergistically in ribosome association.

Having identified that the cytosolic domain of Ysy6p promotes ribosome association and that it is contained in a helix-turn-helix motif which displays conservation across organisims it would be of great interest to determine whether the conserved cytosolic domain of Ysy6p is sufficient to mediate ribosome association. To address this is a peptide could be synthesised corresponding to the conserved cytosolic domain and used to determine if it can compete with Ysy6p for ribosome association.

It has also been shown that ribosome association of Ysy6p is not saturated at endogenous levels. This showed that Ysy6p abundance is not the limiting factor in the process mediating recruitment to ribosomes. In order to further investigate recruitment of Ysy6p to ribosomes an approach which builds on previous work on RAMP4 can be used. It was demonstrated that in vitro translation of the cotranslationaly integrated membrane substrate causes recruitment of RAMP4 to the translocon (Pool, 2009). Similarly, that RAMP4 crosslinks to the transmembrane protein Ii during its cotranslational integration (Schroder et al., 1999). It would therefore be of great interest to determine whether Ysy6p associates with the nascent chain in a similar manner using similar experimental approaches. In addition, this could be used as an opportunity to further investigate the exact conditions which mediate substrate recruitment. Recruitment of RAMP4 has been shown to occur during the emergence of a nascent chain in the ribosomal exit tunnel which is sensed by rpl17 (Pool, 2009). Crosslinking of Ii substrate to RAMP4 however occurs when a hydrophobic cytosolic domain is present in the Sec61p pore and also coincides with the expected nascent chain length for release of the N terminal transmembrane domain of Ii into the lipid bilayer (Devaraneni et al., 2011, Schroder et al., 1999). It has therefore been shown that RAMP4 is recruited to ribosomes upon the emergence of a nascent chain in the ribosomal exit tunnel but that hydrophobic domains are required to exit the ribosomal tunnel before the nascent chain can be crosslinked to the translocon. These observations therefore suggest that RAMP4 is recruited to the ribosome-Sec61 complex followed by close interaction with the nascent chain via hydrophobic domains. Therefore, if crosslinking of Ysy6p is observed to substrate proteins during co-translational translocation further integration constructs could be made with differentially placed hydrophobic or transmembrane domains. This would enable to identify the exact sequence determinants of the nascent protein which mediate recruitment of Ysy6p. For example, it can be asked whether a single hydrophobic domain is sufficient for recruitment of RAMP4/Ysy6p or whether multiple hydrophobic domains are required, whether crosslinking to substrate is concomitant with the presence of a hydrophobic or transmembrane sequence in the translocon or whether release of a transmembrane domain into the lipid bilayer enables crosslinking to Ysy6p.

In summary it has been demonstrated that Ysy6p like RAMP4 is a ribosome associated protein. Furthermore, it has been shown that a cytosolic domain of Ysy6p composed of

a helix-turn-helix motif is involved in promoting ribosome association. These findings together with previous work suggest that Ysy6p and RAMP4 are proteins involved in processes associated with ER bound ribosomes and in particular protein integration in a manner which is conserved across organisms. The exact mechanisms and significance of Ysy6p in these processes will however have to await future work but present exciting perspectives with multiple avenues of investigation.

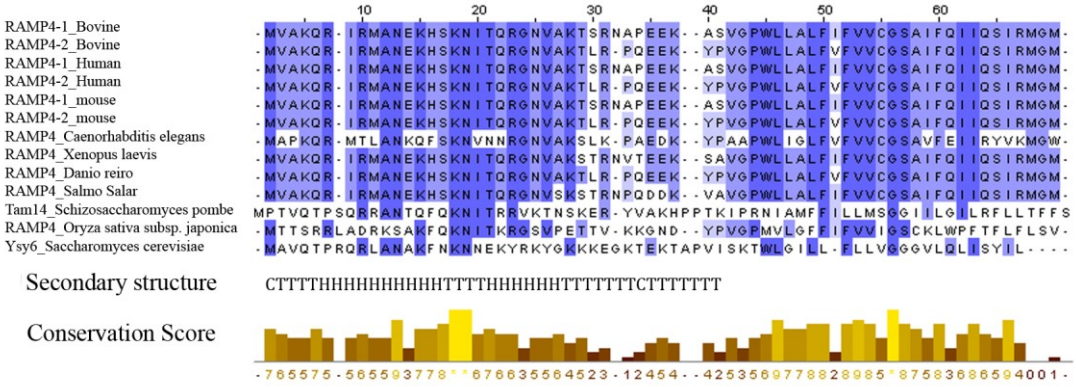
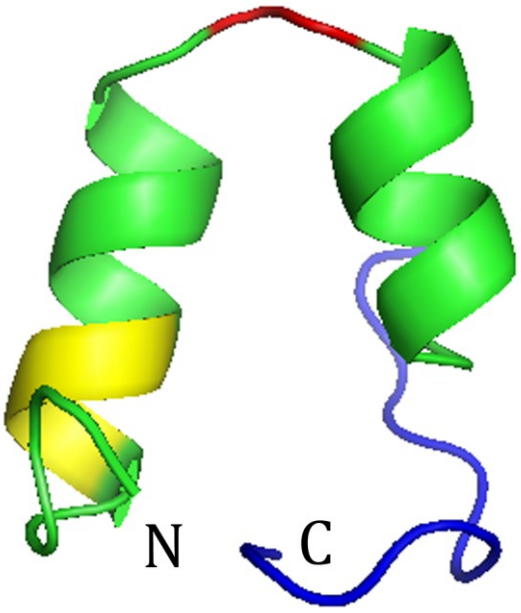


Figure 4.15 Sequence conservation of Ysy6p across organisms. Protein sequences of homologs of Ysy6p obtained from uniprot were compared using clustal omega (Sievers et al., 2011). Sequences were coloured according to sequence conservation. Secondary structure sequence was obtained from the sequence of Ysy6p using Quark *ab initio* modelling for the first 40 amino acids of Ysy6p (C=coil, T=turn, H=Helix).

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>C-coil;H-helix;E-sheet;T-beta turn
MAVQTPRQRLANAKFNKNNEKYRKYGKKKEGKTEKTAPVI
CTTTTHHHHHHHHHHTTTTHHHHHHTTTTTTTCTTTTTTT
12345678901234567890123456789012345678 90
--------10--------20--------30--------40
```

Figure 4.16 Model of the cytosolic domain of Ysy6p. Model of the cytosolic domain of Ysy6p using Quark *ab initio* modelling and coloured using Pymol molecular graphics system (Xu and Zhang, 2012). The model is composed of helix turn helix domain showing conservation (green), the sequence causing loss of cosedimentation is located near the N terminus (yellow), the most highly conserved residues are present in the turn (red) and poorly conserved sequence (blue) is located close to the membrane (blue).

CHAPTER 5:

Investigating the molecular environment of Ysy6p

5.1 Introduction

The mammalian homolog of Ysy6p, RAMP4 was first identified as a protein which is tightly associated with the Sec61p complex and ribosomes (Görlich and Rapoport, 1993). It was demonstrated in chapter 4 that the yeast homolog of RAMP4, Ysy6p, is also ribosome associated. It was also shown that the cytosolic domain of Ysy6p promotes ribosome association. To further investigate how ribosome association is mediated it would be of great interest to explore the molecular environment of Ysy6p. Work carried out by Pool (2009) has demonstrated that RAMP4 can crosslink to the ribosomal protein rpL17 and Yamaguchi et al., (1999) have demonstrated that RAMP4 can cross link to Sec61β. RAMP4 has therefore been shown to be tightly associated to ribosomes due to salt resistance and also spatially intimately associated with the ribosome translocon complex. Crosslinking may therefore determine whether Ysy6p also displays this spatially intimate association.

In chapter 4, Ysy6p was shown to be ribosome associated. However, the ribosome associated material only constitutes a fraction of Ysy6p. This therefore begs the question if there are different pools of Ysy6p which are associated with different partners. Consistent with this possibility are observations from Wang and Dobberstein (1999) who carried out blue native PAGE analysis of RAMP4 and found that RAMP4 exists in an unidentified complex which was shown not to contain the Sec61 complex. Therefore, exploring the molecular environment of Ysy6p may also identify a secondary pool of the protein which has a distinct function from the ribosome associated pool.

In order to explore the molecular environment of Ysy6p a crosslinking approach has been chosen. The first reason for this is that this approach has been productive in the study of RAMP4 (Pool, 2009, Yamaguchi et al., 1999). Secondly, crosslinking can be

carried out on unsolubilised membrane extracts and therefore be carried out in near physiological conditions without the use of detergent.

Blue native PAGE has been used by Wang and Dobberstein (1999) to analyse ER membrane protein complexes which showed that RAMP4 is present in an unidentified complex. However, RAMP4 has also been shown to associate with ribosomes but ribosomal complexes cannot be readily analysed by blue native PAGE due to their size of >4 500 Kda (Görlich and Rapoport, 1993, Hamilton et al., 1971). Ribosomal complexes are for this reason separated on sucrose gradients as described in chapter 4. Secondly, for observation of complexes by blue native PAGE, the complex needs to be resistant to the environment of the gel as well the loading and gel buffers. Therefore complexes which are only loosely associated may remain undetected (Schagger et al., 1994). Lastly, membrane proteins pose an additional problem as complexes need to be able to maintain themselves in the absence of membranes and in an aqueous environment in the presence of detergent. Co-immunoprecipitation techniques face many of the problems encountered by blue native PAGE except that ribosomal complexes can still be identified (Inada et al., 2002). However, as for ribosome cosedimentation non specific binding of proteins to ribosomes increases the probability of false positive results (Serebriiskii et al., 2000).

For these reasons a crosslinking approach has been chosen to investigate the molecular environment of Ysy6p. This allows for complexes present in intact membranes to be covalently linked together. The resulting crosslinks can then be immunoprecipitated under stringent conditions due to the irreversible nature of maintenance of association by the crosslinker. The immunoprecipitate can then be analysed by SDS-PAGE and crosslinking bands analysed by mass spectrometry or by comparative western blotting as previously shown for RAMP4 and Sec61p (Pool, 2009).

5.2 Crosslinking of endogenous Ysy6p

In order to investigate the molecular environment of Ysy6p a crosslinking approach was used. Ysy6p contains numerous lysines throughout the cytosolic domain but no cysteines (Figure 5.1). In order to exploit this, the heterobifunctional crosslinker m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) was used. The maleamide group of MBS reacts with the sulfhydryl groups of cysteines and the NHS ester reacts

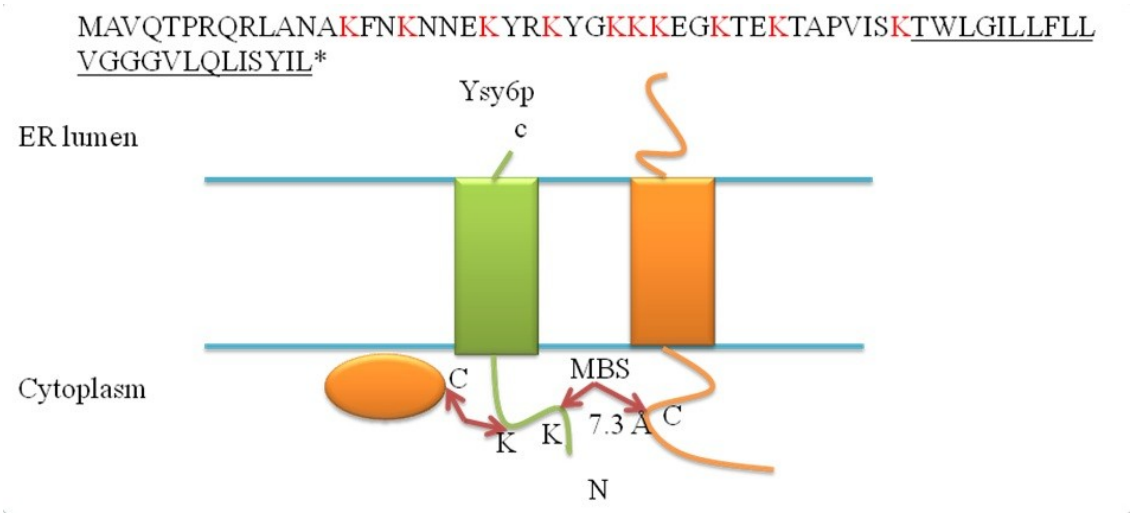

Figure 5.1 Lysine content of Ysy6p and possible MBS linked crosslinking partners. The amino acid sequence of Ysy6p contains multiple lysines (red) in the cytosolic domain but none in the transmembrane domain (black underline). The crosslinking partners must therefore have a cytosolic or transmembrane component.

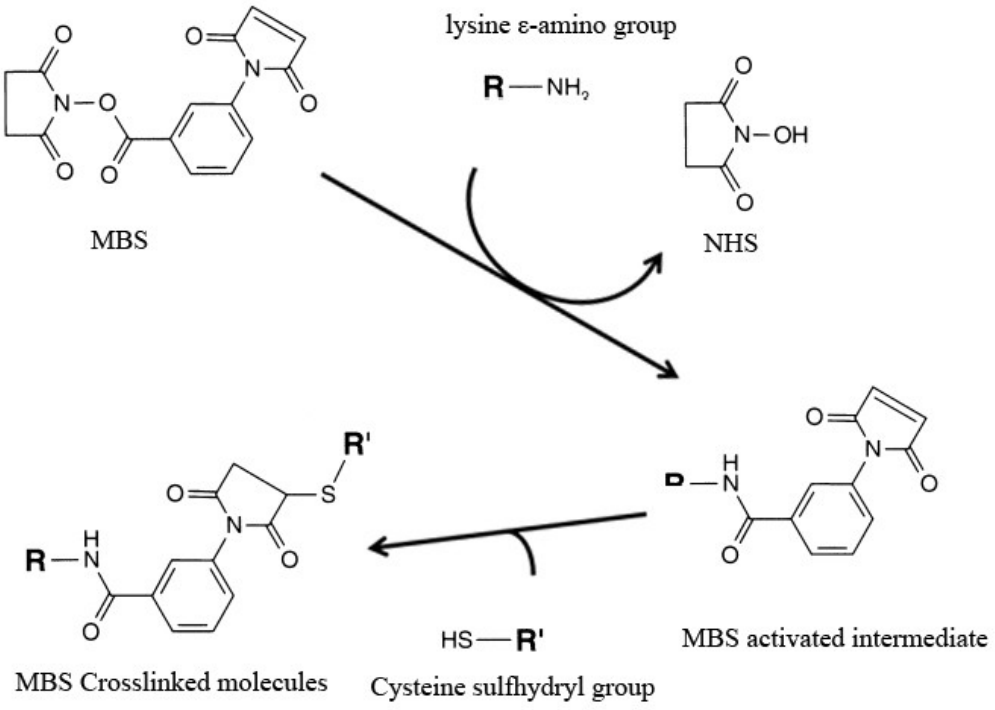

Figure 5.2 MBS crosslinking. MBS firstly reacts with lysine residues to give rise to an active MBS intermediate and causes the release of *N*-Hydroxysuccinimide (NHS). MBS then further reacts with the sulfhydryl group of cysteine residues to yield MBS crosslinked molecules.

With the ε-amino group of lysines (Figure 5.2). NHS esters have a half life of only a few hours in aqueous solutions at physiological pH and can be inactivated by buffers containing primary amines such as Tris. (Hermanson, 2008a). The maleamide group of MBS specifically reacts with cysteines at pHs between 6.5 and 7.5 with cross reactivity to amino groups at higher pH (Hermanson, 2008a). For this reason crosslinking was carried out at a pH of 7.2 buffered using Hepes.

MBS has a spacer arm of 7.3Å and is lipid soluble which means it can readily cross the ER membrane (Hermanson, 2008b). It is advantageous for the crosslinker to be lipid soluble as the extracts used are of crude intact membranes and it is therefore possible that crosslinking may occur between a cytoslic lysine of Ysy6p and a transmembrane segment of the crosslinking partner. The spacer arm length of 7.3 Å is at the shorter end of the crosslinker arm lengths available. Crosslinkers with shorter arm lengths, such as 1,5-Difluoro-2,4-dinitrobenzene (DFDNB) are more suited for studying promiscuous interactions such as inter-subunit associations (Kornblatt and Lake, 1980).

Crosslinking was carried out using crude membrane preparations from the lysate cosedimenting at between 1 200g and 16 000g referred to here as the P16 membrane preparation. The P16 membrane preparation includes the majority of ER associated material as well as nuclear and mitochondrial material (Frey et al., 2001). Lysis was performed in low salt (LS) buffer in order to minimise potential denaturation of protein. Crosslinking was carried out as described in section 2.8.8 using MBS to a final concentration of 180 μM MBS and 900 μM (Figure 5.3). This gave rise to the detection of two crosslinking products of 32 (a) and 28 kDa (b). The lower crosslink is the most efficiently crosslinked product with crosslinking at 180 μM MBS. The upper crosslink is not efficiently formed at 180 μM as its intensity was increased when treated with 900 μM MBS.

In order to explore whether the reported genetic interaction between *YSY6* and *EMC5* had an effect on the molecular environment of Ysy6p, the crosslinking profile of Ysy6p was investigated in the *Δemc5* strain (Schuldiner et al., 2005, Ito et al., 2001). Differences in the crosslinking profile between the *WT* and *Δemc5* would demonstrate that Emc5p affects the molecular associations of Ysy6p. However, no change in the crosslinking profiles between the *WT* and *Δemc5* deletion strains could be observed

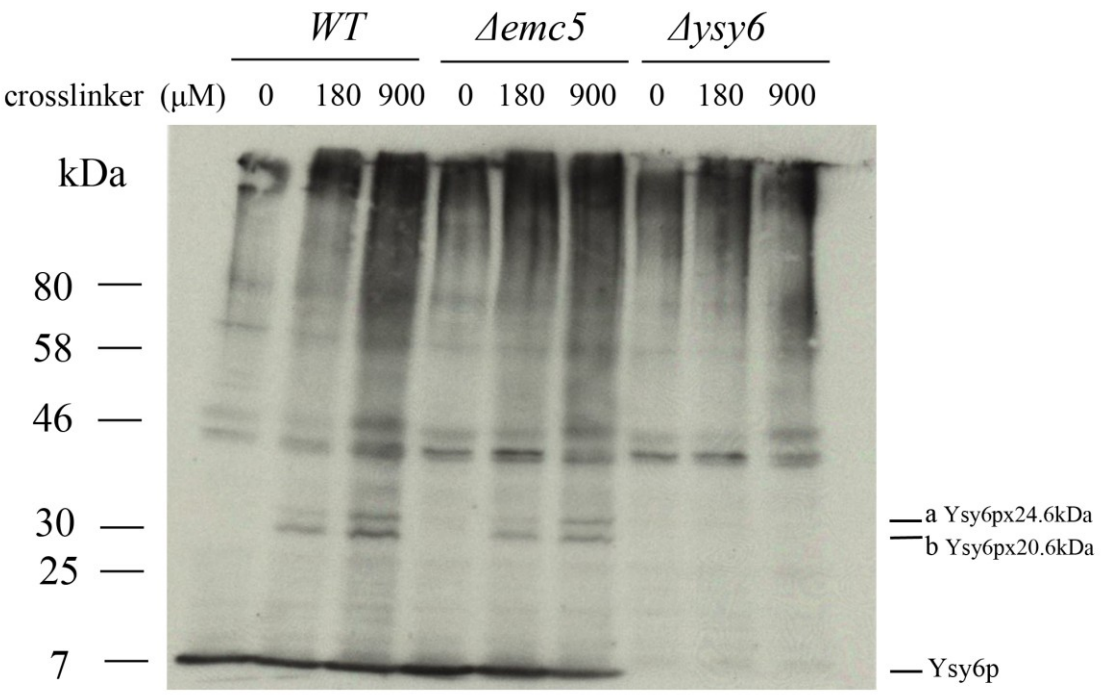

Figure 5.3 Cross-linking of Ysy6p with MBS The membrane enriched P16 fraction prepared from the *BY4742 WT, Δemc5 Δysy6* strains propagated in YPD media were treated with MBS to the stated final concentration at 24°C for 20 minutes. 5 OD_{600} cell unit equivalent of P16 membrane preparations were loaded onto 10% SDS-PAGE gel and western blotting using the anti Ysy6p antibody. Representative of 2 repeat experiments.

with crosslinking products (a) and (b) present in the *Δemc5* strain with similar intensities and no other crosslinking specific products observed.

5.3 Crosslinking using the Ysy6p-opsin overexpression construct gives rise to a physiologically relevant crosslinking profile.

Having established an endogenous crosslinking pattern for Ysy6p the next step was to try and immunoprecitpitate the crosslinks for identification by mass spectrometry. Due to the relative weakness of the signal of the Ysy6p crosslinking products in relation to total Ysy6p it was decided that identification of the crosslinking products would be best achieved by an overexpression approach. Ysy6p was therefore over-expressed using the Ysy6p-opsin construct under the control of the *MET25* promoter previously described in chapter 4.

Crosslinking was carried out as described in section 2.8.8 using MBS to a final concentration of 900 μM with membranes prepared from cell cultures of the Δ*ysy6* strain expressing Ysy6p-opsin. The crosslinking products were then analysed on 12.5% SDS-PAGE gel followed by western blotting with the Ysy6p antibody. A 12.5% SDS-PAGE gel was used instead of a 10% SDS-PAGE gel in order to obtain a better resolution of crosslinks in the 20 to 40 kDa range. As shown in figure 5.4 crosslinking gave rise to crosslinking products which included three strong bands of 32 kDa (A), 28 kDa (B) and 19 kDa (C). In addition, other fainter bands of 31kDa (*1), 29 kDa (*2) and 20 kDa (*3) bands could also be detected. The 32kDa (A) and 28 kDa (B) bands are consistent with the size of the previously observed crosslinking products for endogenous Ysy6p (figure 5.3).

In order to further investigate the appearance of an additional strong 19 kDa (C) crosslinking product and the additional faint crosslinking products crosslinking reactions carried from P16 membrane preparations from strains expressing either endogenous Ysy6p or Ysy6p-opsin were analysed by SDS-PAGE concomitantly. It was hypothesised that the additional bands observed in the over-expression construct could be due to several possibilities. Firstly, that the additional crosslinks are specific for over-expression of Ysy6p-opsin. Secondly, the additional bands did not appear when crosslinking endogenous Ysy6p because they had undergone a degredative event. This

hypothesis was put forward as degradation of Ysy6p had already been observed to cause loss of cosedimentation with ribosomes as discussed in chapter 4. Lastly, that the additional bands were only detected as a consequence of proteolysis of the higher molecular weight crosslinks. Crosslinking was performed as described in section 2.8.8 using MBS to a final concentration of 900 μM and P16 membrane preparations made from the *WT BY4742*, *CST211* and *BY4742* Δ*ysy6* strains propagated in media lacking methionine and the *BY4742* Δ*ysy6* strain transformed with *pRS315-Ysy6N* propagated in media lacking methionine and leucine. Crosslinking reactions were incubated at 37°C for 20 minutes. As previously described, strain *CST211* containes point mutations to vacuolar proteinases which was shown in Chapter 4 to inhibit degradation of Ysy6p. Crosslinking reactions were analysed on 12.5% SDS-PAGE and western blotting with the Ysy6p antibody. As shown in figure 5.5, the *WT* strain expressing endogenous Ysy6p gave rise to three crosslinker specific bands of 32 kDa (A), 28 kDa (B) and 19 kDa (C). The Ysy6-opsin expressing strain gave rise to bands of similar size corresponding to the 32 kDa (A), 28 kDa (B) and 19 kDa (C) bands observed in the *WT* strain. In addition, the previously observed bands of 31 kDa (*1), 29 kDa (*2) and 20 kDa (*3) bands were also observed. The triple protease mutant *CST211* also gave rise to crosslinker specific bands observed in the *WT* strain of 19 kDa (A), 28 kDa (B) and 32 kDa (C). However, crosslinking of the *CST211* strain also gave rise to additional bands of weaker intensity (.) of 40 kDa, 34 kDa, 33 kDa and 17 kDa which are not present in the other strains. The data therefore suggest that the 19 kDa band was initially not observed in figure 5.3 due to inefficient formation of the crosslink. Furthermore, the fact that the 19 kDa band is detectable for the endogenous strain only when crosslinking is performed at 37°C suggests that the crosslinking is more efficient when performed at 37°C. Consistent with this is the observation that the relative intensities of the previously inefficiently formed 31 kDa and 29 kDa bands are increased when crosslinking is performed at 37°C. The triple protease mutant *CST211* strain showed the formation of additional crosslinks which were not detected either in the strain overexpressing Ysy6p-opsin or the *WT BY4742* strain. This suggests either that the crosslinking profile is affected by the action of proteinases post lysis or alternatively the deletion of proteinases affects factors which associate with Ysy6p.

Taken together the data demonstrate that crosslinking with MBS results in the formation of three crosslinks to endogenous Ysy6p the abundance of which can be

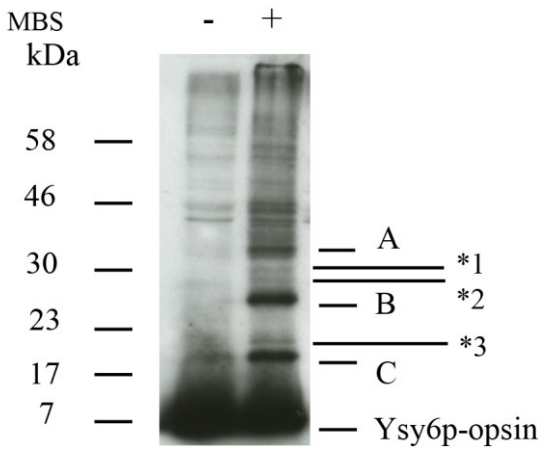

Figure 5.4 Crosslinking of Ysy6p-opsin with MBS The membrane enriched P16 fraction was prepared from the *BY4742* Δ*ysy6* strain transformed with *pRS315-Ysy6N* plasmid and propagated in SD media lacking leucine and methionine. Crosslinking was carried out by MBS treatment to a final concentration of 900 μM (lane 2) or mock treated with DMSO (lane 1) and incubated at 24°C for 20 minutes. 2.5 OD_{600} of cell culture equivalent was then loaded onto a 12.5% SDS-PAGE gel followed by western blotting with the anti Ysy6p antibody.

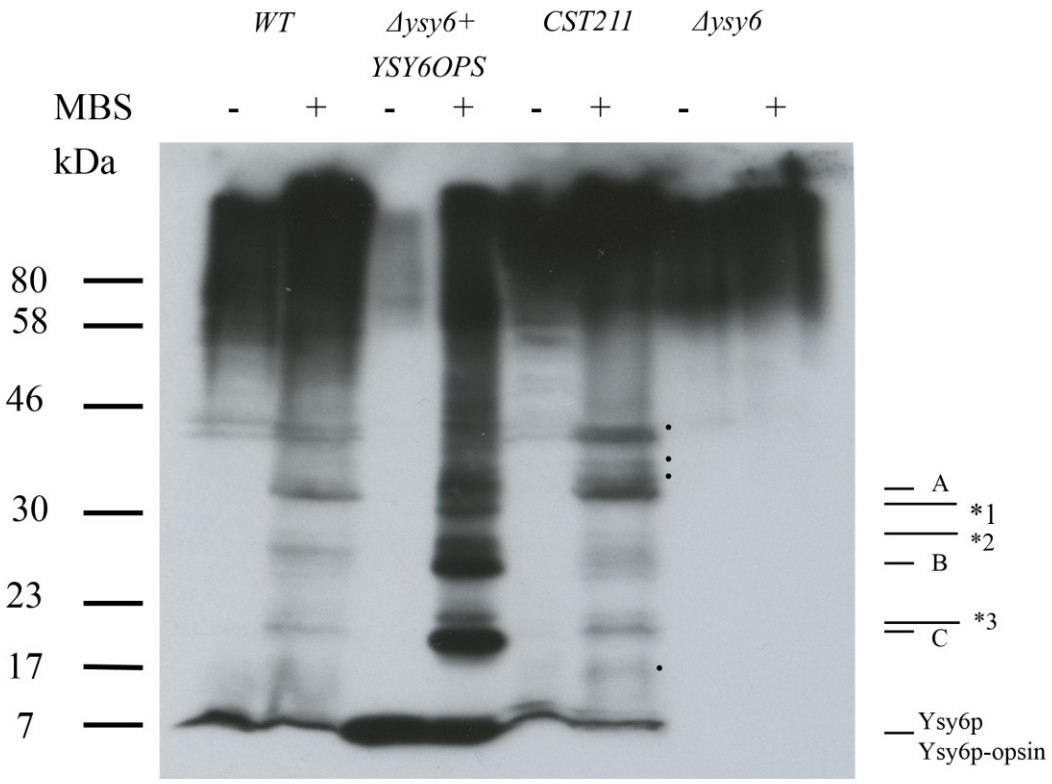

Figure 5.5 Comparison of the crosslinking profile across the *BY4742 WT*, *CST211* and the Δ*ysy6* strain expressing Ysy6p-opsin. Membrane enriched P16 fractions were made from strains *BY4742 WT*, *CST211*, *BY4742* Δ*ysy6* and *BY4742* Δ*ysy6* expressing Ysy6p-opsin (YSY6OPS) in SD media lacking methionine or lacking leucine and methionine as appropriate. P16 fractions were treated with MBS to a final concentration of 900 μM (+) or mock treated with DMSO (-) and incubated at 37°C for 20 minutes. 0.5 OD_{600} units of cell culture equivalent of the sample prepared from the Δ*ysy6* strain expressing Ysy6p-opsin and 5 OD_{600} units of cell culture equivalent of all other samples were loaded onto a 12.5% SDS-PAGE gel followed by western blotting with anti Ysy6p antibody.

increased by over-expression of Ysy6p-opsin. The crosslinks are of 32 kDa, 28 kDa and 19 kDa and Ysy6p is a 7.4 kDa protein which suggests that the crosslinking partners are of approximately 25 kDa, 21 kDa and 11 kDa respectively. The Ysy6p-opsin overexpression construct gives rise to additional crosslinking products of 31 kDa, 29 kDa and 20 kDa which are inefficiently formed when crosslinking is performed at 24°C but increase in abundance when crosslinking is carried out at 37°C. This suggests that crosslinking with Ysy6p-opsin gives rise to a more physiologically relevant crosslinking profile when performed at 24°C than 37°C.

5.4 Crosslinking of Ysy6p with DSS

In order to identify more crosslinking products the crosslinker disuccinimidyl suberate (DSS), was also tested. DSS is a homobifunctional lysine crosslinker and therefore, contrary to MBS, can potentially crosslink Ysy6p to proteins which do not contain cysteines. It has already been demonstrated that the mammalian homolog of Ysy6p, RAMP4, crosslinks to rplL17 using MBS (Pool, 2009). However, the yeast homolog of rpL17, rpl17, is a 20.5 kDa protein which contains no cysteines but multiple lysines (Cherry et al., 2012). In addition, DSS has an arm length of 11.3Å in comparison to 7.3Å for MBS (Hermanson, 2008b, Hermanson, 2008c). This theoretically means that DSS is less spatially constrained than MBS and therefore increases the chance of detecting any interaction between Ysy6p and Rpl17p. Crosslinking with DSS was performed using the P16 membrane fraction prepared from the *BY4742* Δ*ysy6* strain transformed with *pRS315* or *pRS315-YSY6N* and propagated in SD media lacking leucine and methionine as described in section 2.8.8. The P16 was treated with DSS to a final concentration of 0.4 mM, 0.2 mM 0.1 mM DSS or mock treated with DMSO. As shown in figure 5.6 treatment with DSS gave rise to a strong crosslinking product of 20 kDa at all concentrations of crosslinker. Other crosslinking bands were observed (*) but the faintness of these bands relative to the total Ysy6p-opsin render characterisation of these crosslinks difficult. Crosslinking with DSS therefore gives rise to a strong crosslinking product of 20 kDa. Ysy6p-opsin was calculated from figure 4.9 to be of 9.7 kDa and therefore that Ysy6p-opsin crosslinks to protein of approximately 10 kDa. The size of the crosslinking partner is therefore inconsistent with the size of rpl17p and therefore no crosslinking to rpl17p could be observed.

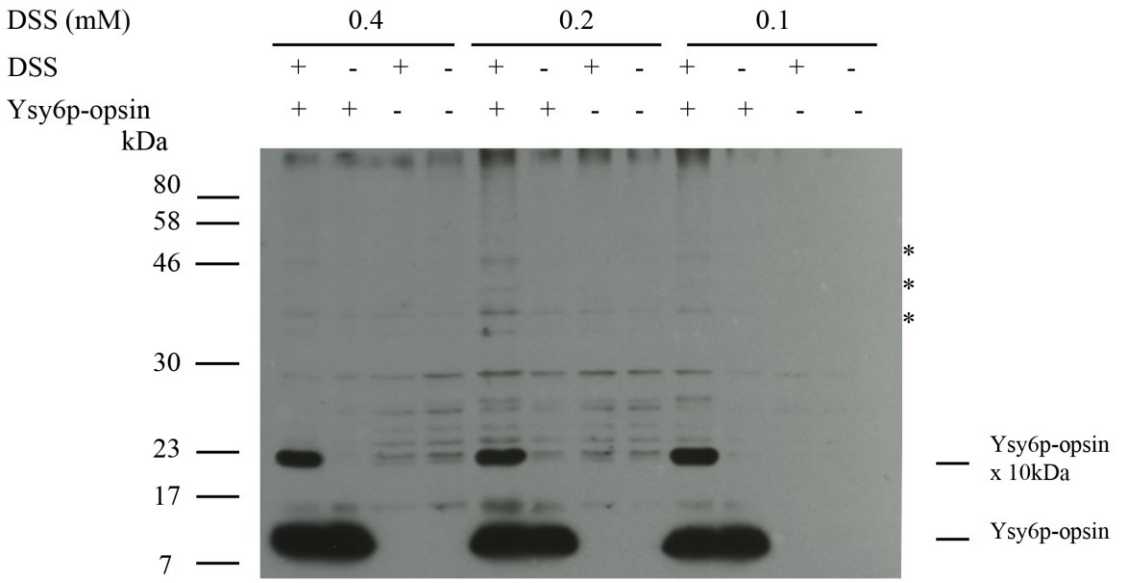

Figure 5.6 Crosslinking Ysy6p with DSS. Membrane enriched P16 fractions were prepared from the *BY4742* Δ*ysy6* strain transformed with either plasmid *pRS315* or *pRS315-YSY6N* and propagated in SD media lacking leucine and methionine. The membrane preparation was then crosslinked with the stated final concentration of DSS or mock treated with DMSO and incubated at 24°C for 20 minutes. 5 OD_{600} units of cell culture equivalent was then loaded onto a 12.5% SDS-PAGE gel followed by western blotting with the anti opsin antibody.

5.5 Immunoprecipitation of Ysy6p-opsin using the Ysy6 antibody.

In order to attempt identification of the Ysy6p crosslinking partners an approach previously used by Pool (2009) consisting of immnoprecipitation of crosslinking products and mass spectrometry. The first stage was to investigate whether the Ysy6p antibody was suitable for immunoprecipitation. To do this the P16 membrane extract was prepared from the *BY4742 Δysy6* strain transformed with *pRS315-YSY6N* or *pRS315* and propagated in SD media lacking methionine and leucine. Ysy6p-opsin was then immunoprecipitated using the Ysy6p antibody as described in 2.8.9 followed by analysis by SDS-PAGE and western blotting. As can be seen in figure 5.7, this gave rise to a band of ca. 12 kDa being immunoprecipitated which is consistent with the expected size of Ysy6p-opsin when resolved on 15% SDS-PAGE gels. Furthermore, this band is absent in immonprecipitations performed in the absence of P16 or Ysy6p-opsin, thereby demonstrating the specificity of the band to membrane preparations containing Ysy6p-opsin. Ysy6p-opsin was therefore immunoprecipitated.

In order to optimise the immunoprecipitation conditions saturation of the anti Ysy6p antibody was investigated. This was investigated by carrying out immunoprecipitations using a fixed amount of Ysy6p antibody and decreasing amounts of membrane preparations. As shown in figure 5.8 the amount of whole serum Ysy6p antibody was kept constant at 5 µl per IP whereas two-fold dilutions of membrane preparations were made to yield 100 OD_{600} to 12.5 OD_{600} of cell culture equivalent per IP. The binding of Ysy6p-opsin to the Ysy6p antibody increases with increased membrane extract until a load of 50 OD_{600} of cell culture equivalent. This indicates that the antibody saturation is of 10 OD_{600} per 1 µl under these conditions. In addition, comparison of the signal from the precipitate and supernatant suggests that the efficiency of the immunoprecipitation is of around 5 to 10%.

The next step was to try and increase the efficiency of the immunoprecipitations. It was hypothesised that the high salt concentration of the IP wash buffer B could lead to dissociation of Ysy6p from the antibody due to disruption of electrostatic interactions. To test this, immunoprecipitates were carried out as described in 2.8.9 but washed either with Buffer B containing 0.5M NaCl or 0.14M NaCl. However, no observable effect could be observed on the immunoprecipitation efficiency (Figure 5.9).

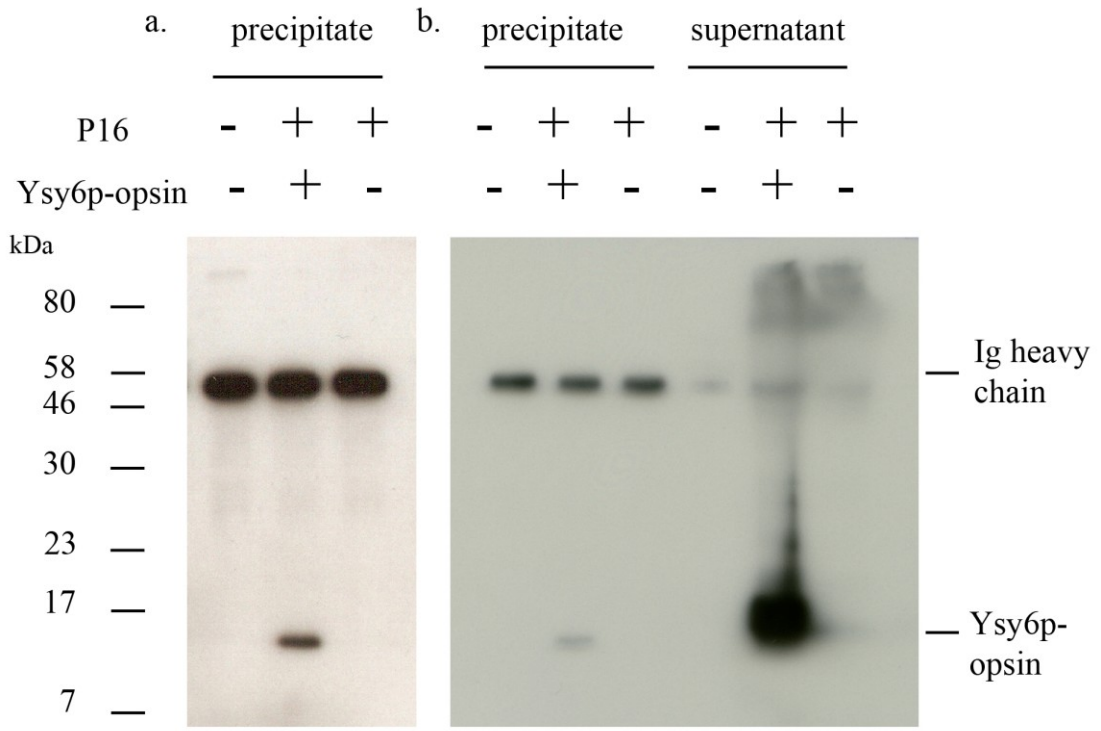

Figure 5.7 Immunoprecipitation of Ysy6p-opsin Immunoprecipitations were carried out using 650 OD_{600} units of cell culture equivalent of the P16 membrane enriched fraction prepared from the *BY4742 Δysy6* strain transformed with either *pRS315* or *pRS315-YSY6N* and propagated in SD media lacking leucine and methionine. Ysy6p-opsin was immunoprecipitated using 4 µl Ysy6p antibody prebound to 60 µl 50% (v/v) protein A sepharose. The samples were analysed by loading 7.5% of the supernatant and precipitate onto a 15% SDS-PAGE gel followed by western blotting with the Ysy6p antibody. A long (a) and short exposure (b) of the western blot is shown.

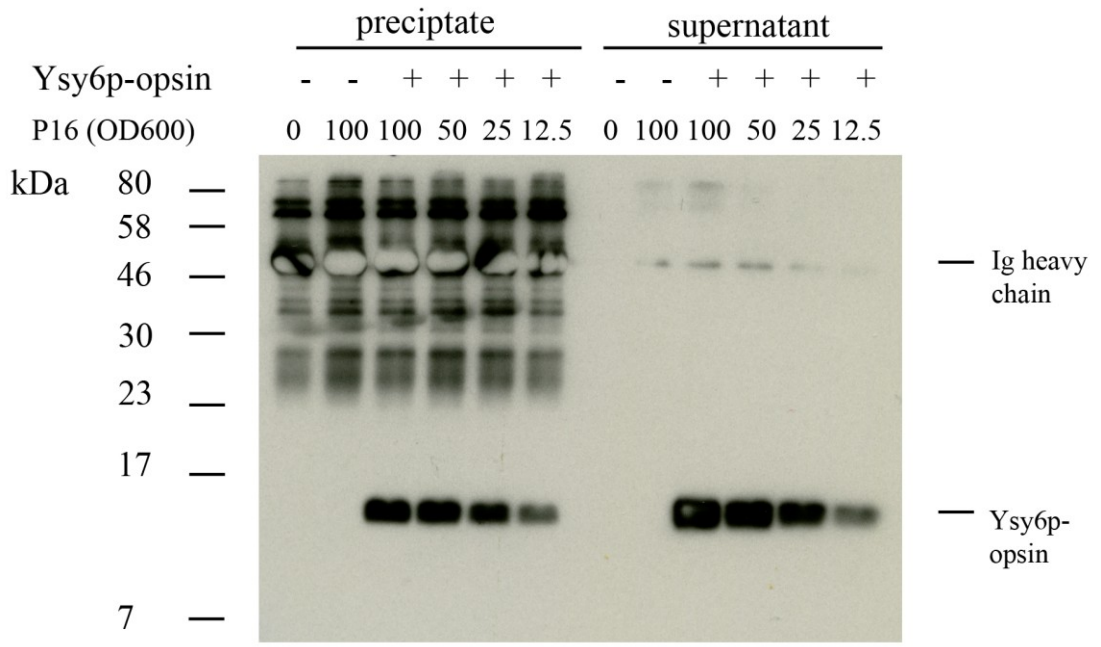

Figure 5.8 Ysy6p antibody saturation Immunoprecipitations were carried out using the stated amount P16 membrane enriched fraction prepared from the *BY4742* Δ*ysy6* strain transformed with either *pRS315* or *pRS315-YSY6N* and propagated in SD media lacking leucine and methionine. Ysy6p-opsin was immunoprecipitated using 20 µl of 50% (v/v) protein A sepharose beads prebound to 5 µl Ysy6p antibody. Samples were analysed by loading 1% of the supernatant and 10% of the precipitate onto a 15% SDS-PAGE gel followed by western blotting using the Ysy6p antibody.

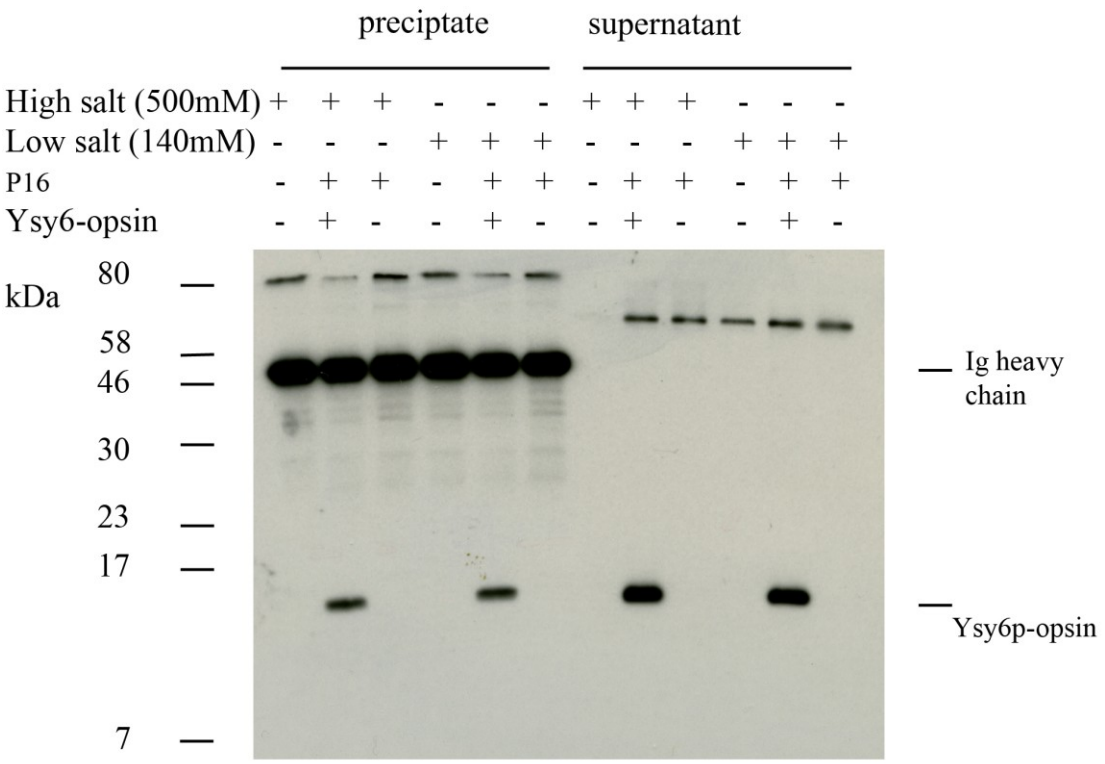

Figure 5.9 Comparing the effect of salt concentration in Buffer B on the efficiency of immunopreciptation. Immunoprecipitations were carried out using 100 OD_{600} units of cell culture equivalent of P16 membrane enriched fraction prepared from the *BY4742* Δ*ysy6* strain transformed with either *pRS315* or *pRS315-YSY6N* and propagated in SD media lacking leucine and methionine. Immunoprecipitations were carried out using 20 µl of 50% (v/v) protein A sepharose and 5 µl Ysy6p antibody not prebound to protein A sepharose. The immunoprecipitates were washed with either high salt or low salt buffer B as specified. Samples were analysed by loading 1% of the total supernatant and 10% of the precipitate onto a 15% SDS-PAGE gels followed by western blotting using the Ysy6p antibody.

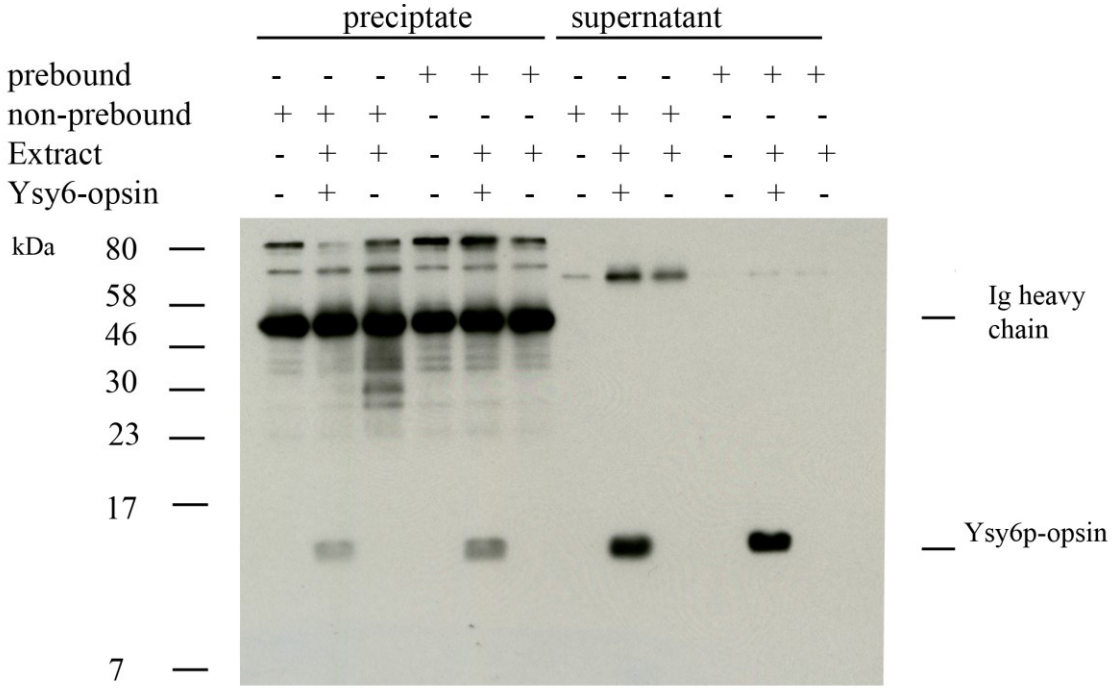

Figure 5.10 Comparing the efficiency of immunoprecipitation of the Ysy6p antibody when bound first to protein A sepharose or the antigen. Immunoprecipitations were carried out using 100 OD_{600} units of cell culture equivalent of P16 membrane enriched fraction prepared from the *BY4742* Δ*ysy6* strain transformed with either *pRS315* or *pRS315-YSY6N* and propagated in SD media lacking leucine and methionine. Immunoprecipitations were carried out using 20 µl of 50% (v/v) protein A sepharose beads and 5 µl Ysy6p antibody. The antibody was either prebound for one hour to protein A sepharose followed by addition to the membrane preparation or added directly to the lysate and bound to protein A sepherose for 1 hour after overnight incubation. Samples were analysed by loading 1% of the supernatant and 10% of the precipitate onto a 15% SDS-PAGE gel followed by western blotting using the Ysy6p antibody

In the methodology used so far the antibody was bound to the antigen prior to binding to protein A sepharose or inversely the antibody was first bound to protein A sepharose followed by binding to the antigen. To test if this affected the immunoprecipitation efficency immunopreciptations were carried out as described in 2.8.9 using both methodologies for binding the antibody to the antigen. The antibody was either added to the lysate and left overnight to bind at 4°C followed by binding to beads for one hour or the antibody was bound to beads for one hour followed by addition to the lysate and left overnight. As shown in figure 5.10 prebinding of the anitbody to beads was marginally more efficient than the converse in immunoprecipitating Ysy6p-opsin.

5.6 Immunoprecipitation of the crosslinking products.

Having carried out optimisation of the crosslinking and the immunoprecipitation, the next step was to immunoprecipitate the crosslinking products. Crosslinking was carried out using MBS as previously described in section 2.8.8 but using the P16 membrane preparations at a concentration of 2.5 OD_{600}/µl of cell culture equivalent and scaled up to use 100 OD_{600} of cells per reaction. The reaction was quenched with cysteine to a final concentration of 5 mM. As can be seen in figure 5.11 the three major crosslinks previously observed in figure 5.4a were Immunoprecipitated with Mw of 32 kDa, 28 kDa and 19 kDa. Silver staining of the immunoprecipitate (Figure 5.11 b) shows that two of the crosslinking products are masked by the light chain. The 19 kDa crosslink was however detectable by silver staining. This band was sent for analysis by mass spectrometry but the only peptide hit was Hho1p which is a histone protein of 27 kDa (Cherry et al., 2012, Ushinsky et al., 1997). Due to the size of the protein which is bigger than the crosslink itself it is most likely to be a contaminant. The masspectrometry therefore failed to identify Yy6p-opsin or its crosslinking partner. However, the crosslinking products were successfully immunoprecipitated.

5.7 Quantification of the amount of Ysy6p-opsin in sample.

Since the amount of Ysy6p immunoprecipitated was not readily detectable by silver staining the amount of Ysy6p-opsin immunoprecipitated when using 50 OD_{600} cell

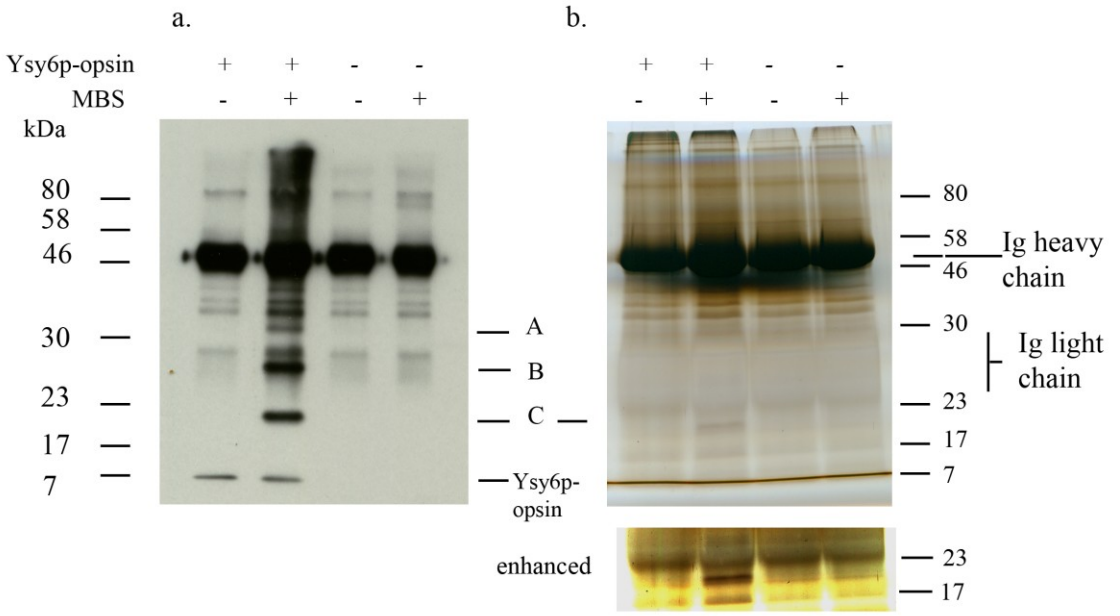

Figure 5.11 Immunoprecipitation of the Ysy6p-opsin crosslinks. Immunoprecipitations were carried out using 100 OD_{600} cell culture equivalent of P16 membrane enriched fraction prepared from the *BY4742* Δ*ysy6* strain transformed with either *pRS315* or *pRS315-YSY6N* and propagated in SD media lacking leucine and methionine. P16 membrane preparations at a final concentration of 2.5 OD_{600}/µl were treated with MBS to a final concentration of 500 µM (+) or mock treated with DMSO (-) and incubated at 24°C for 20 minutes. Crosslinks were immunoprecipitated using 20 µl of 50% (v/v) protein A sepharose beads prebound to 5 µl Ysy6p antibody. Samples were then analysed by loading 1% of the total supernatant and 10% of the precipitate were loaded onto 12.5% SDS-PAGE gels and followed by western blotted using the Ysy6p antibody (a) or silver staining (b). Silver staining shown as scanned or altered by increased contrast (enhanced).

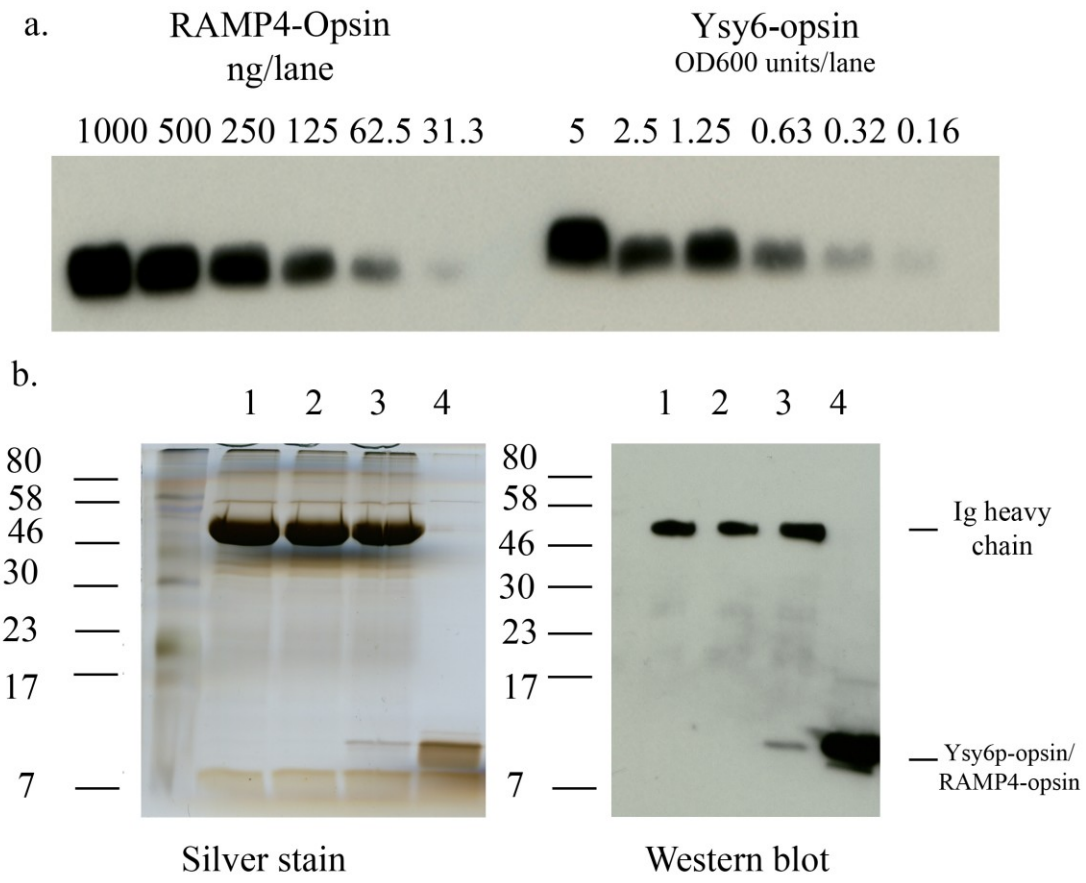

Figure 5.12 Quantification of the Ysy6p-opsin precipitate (a) comparative western blotting with the opsin antibody by loading specified amounts of purified RAMP4-opsin or membrane preparations of the Δ*ysy6* strain expressing Ysy6p-opsin. (b) immunoprecipitation of Ysy6p-opsin using the Ysy6p antibody using 20 µl of 50% (v/v) protein A sepherose beads prebound to 5 µl Ysy6p and using either no P16 membrane preparation (1), 100 OD_{600} cell culture equivalent of membranes prepared from the *BY4742* Δ*ysy6* transformed with *pRS315* strain (2) or the *BY4742* Δ*ysy6* transformed with *pRS315-YSY6N* expressing Ysy6p-opsin (3). Samples were analysed by silver staining and (C) western blotting by loading 90% and 10% of the immunoprecipitate respectively onto 12.5 % gels. 625 ng of RAMP4-opsin was used as a positive control for silver staining and 62.5 ng for western blotting (4).

culture equivalent of membrane preparations and 5 µl of Ysy6p antibody was estimated. This was done by quantifying the amount of Ysy6p-opsin present in the P16 membrane preparation. In order to quantify the amount of Ysy6p-opsin comparative western blotting with anti opsin antibody of known amounts of purified RAMP4-opsin and membrane preparations of the *BY4742* Δ*ysy6* strain expressing Ysy6p-opsin. RAMP4-opsin was used as it was hypothesised, being the mammalian homolog of Ysy6p-opsin and to contain the same opsin tag, to have a similar silver staining efficiency to Ysy6p-opsin. From this western blotting approach it was estimated that 0.4 OD_{600} units of cell culture equivalent of membrane preparations corresponded to 62.5 ng of RAMP4-opsin (figure 5.12a). The total amount of Ysy6p-opsin was calculated by equation 5.1.

Equation 5.1

$$\boldsymbol{mass\ Ysy6p-opsin\ /50\ OD_{600}} = \frac{\boldsymbol{50 \times mass\ RAMP4OPG \times Mw\ Ysy6OPG}}{\boldsymbol{Mw\ RAMP4OPG \times OD_{600} loaded}}$$

$$= \frac{\boldsymbol{50 \times (62.5 \times 10^{-9}) \times\ 9734}}{\boldsymbol{8796 \times 0.4}} = \boldsymbol{8.64\ ug}$$

In addition to this, immunopreciptations are saturated at 50 OD_{600} with an efficiency of approximately 1 to 10%. The expected immunoprecipitate was therefore between 80-800 ng. When Ysy6p-opsin was immunoprecipitated a band corresponding Ysy6p-opsin is visible (Figure 5.12 b). However, when the signal of Ysy6p-opsin is compared 600 ng RAMP4-opsin it is clear that the Ysy6p-opsin is fainter and consistent with the lowest expected value of 80ng (Figure 5.12b). This therefore suggests that the amount of Ysy6p-opsin that can be immunoprecipitated makes identification by mass spectrometry challenging.

5.8 Ysy6p does not crosslink but genetically interacts with *SEC71*

In an effort to identify the crosslinking partners for endogenous Ysy6p an alternative screening approach was attempted. Loss of crosslinking in a deletion strain of a gene encoding a candidate crosslinking partner would suggest the identity of a particular factor which could then be confirmed using a comparative western blotting approach with the relevant antibodies. Candidates were identified by screening the Saccharomyces Genome Database (SGD) and chosen on the basis of size of between 20 and 30 kda, presence of cysteines and involvement in processes of interest such as protein integration and ERAD (Cherry et al., 2012). This methodology was restricted due to the fact that only non-essential genes could be investigated. This yielded four possible candidates namely Ubc7p (18.5 k Da), Sec71p (24 kDa), Sec72p (21.6 kDa) and Cue1p (22.7 kDa).

Sec71p and Sec72p are non-essential subunits of the Sec63/Sec62 complex which is associated with the Sec61p complex and required for the integration and translocation of post-translationally as well as co-translationally targeted substrates at the ER (Brodsky and Schekman, 1993). For example it has been shown that the post-translationally targeted protein CPY is compromised in its translocation in both Δ*sec71* and Δ*sec72* cells (Feldheim and Schekman, 1994, Feldheim et al., 1993). Furthermore, it has been shown that Sec72p is a cytosolic protein whose recruitment to the Sec63/62 complex is dependent on the presence of the transmembrane protein Sec71p. Therefore loss of crosslinking in a Δ*sec71* strain could be attributable to loss of either Sec71p or Sec72p in the Sec62/63 complex.

Cue1p and Ubc7p function in ERAD and are non essential factors of the Doa10p and Hrd1p complexs (Carvalho et al., 2006, Ravid et al., 2006). Cue1p promotes recruitment of Ubc7p to the ER membrane (Kostova et al., 2009, Biederer et al., 1997). Ubc7p is an E2 ubiquitin ligase enzyme which transfers ubiquitin to ERAD substrates in a manner which is mediated to the Doa10p and Hrd1p E3 ubiquitin ligases (Bays et al., 2001b, Swanson et al., 2001).

As shown in figure 5.13 crosslinking was carried out with P16 membrane preparations expressing endogenous Ysy6p with MBS to a final concentration of 900 μM as described in 2.8.8. As shown in figure 5.13, all investigated deletion strains gave rise to

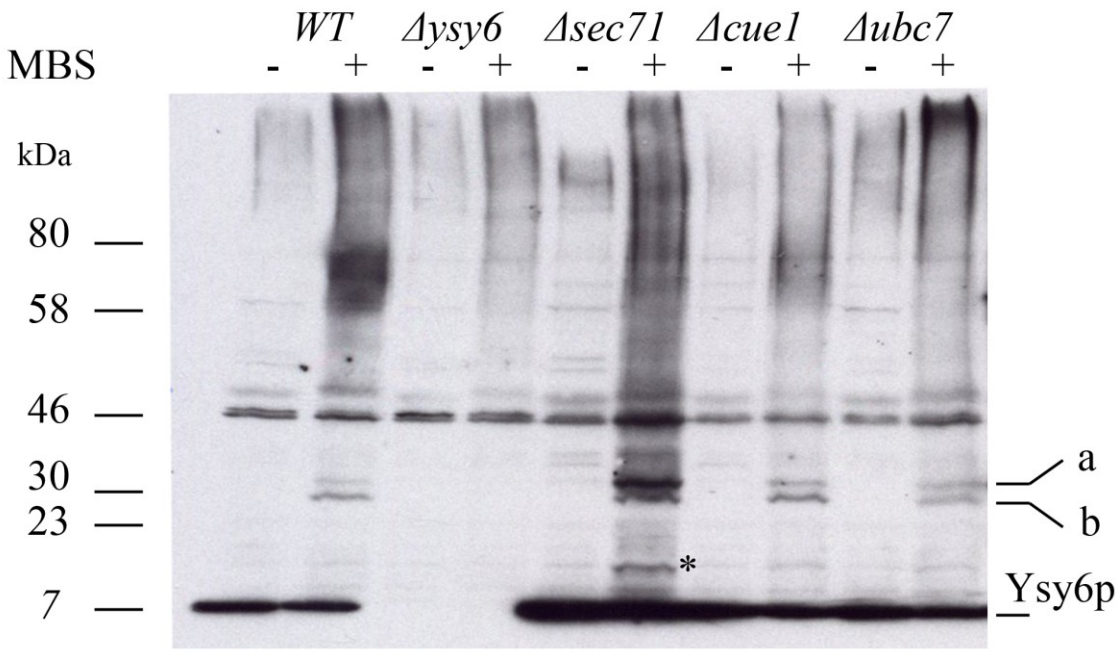

Figure 5.13 Screening deletion strains for loss of crosslinking to Ysy6p. P16 membrane preparations were prepared from indicated deletion strains from the *BY4741* background propagated in YPD media. P16 membrane preparations were treated with MBS to a final concentration of 900 μM (+) or mock treated with DMSO (-) and incubated at 24°C for 20 minutes. Samples were analysed by loading 5 OD_{600} units of cell culture equivalent onto a 10% SDS-PAGE gels and analysed by western blotting using the anti Ysy6p antibody. Three crosslinking products are indicated as "a", "b" and "*". Representative of 2 repeat experiments.

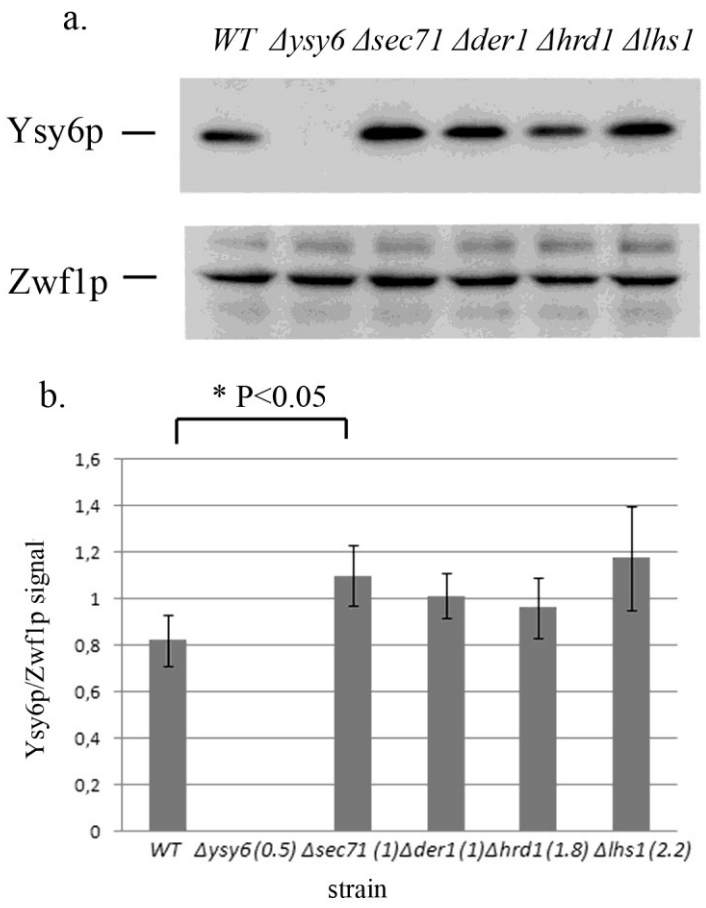

Figure 5.14 Quantification of Ysy6p in strains with induced UPR. (a) Cells were propagated to mid log phase in rich media and lysed using NaOH lysis followed by TCA precipitation. 0.15 OD_{600} units of cell culture equivalent were then loaded onto a 12.5% SDS-PAGE gel and western blotted using the Ysy6p antibody followed by western blotting using the Zwf1p antibody. (b) The intensity of the Zwfp and Ysy6p signals were quantified using AIDA. The Ysy6p signal intensites were then normalised to Zwf1p and the mean and SEM calculated (n=3). The differences between the *WT* and the query strains were tested for significance using a two-tailed unpaired t-test and the P value determined. Strains arranged in order of increasing UPR induction (x=$\log_2$ UPR induction) as determined by Jonikas *et al.,* (2009).

crosslinking products of 32 kDa (a) and 28 kDa (b) in the *WT* strain as previously observed in Figure 5.5. As expected the crosslinking products were absent in the Δ*ysy6* strain. Crosslinking in membrane preparations from the deletion strains Δ*sec71*, Δ*cue1* and Δ*ubc7* gave rise to persistence of the 32 kDa (a) and 28 kDa (b) crosslinking products. This demonstrates that the crosslinking partners of Ysy6p are not Sec71p, Sec72p, Cue1p or Ubc7p. The Δ*sec71* strain showed an additional crosslink of approximately 15 kDa (*). It is uncertain as to whether this band is specific to the Δ*sec71* strain as the level of Ysy6p appears increased relative to the other strains in this strain and runs concomitantly with a cross-reacting band which is not specific to MBS treated samples. The 15 kDa crosslinking product is however consistent with the previously observed inefficiently fromed crosslinking product of 19 kDa (c) in figure 5.5. The difference in apparent molecular weight being attributable to the difference in SDS-PAGE gel percentages used to resolve the proteins. Due to the fact that the level of Ysy6p in the Δ*sec71* strain appeared increased relative to the *WT* strain this apparent difference was further investigated using a quantitative western blotting approach. In addition deletion of Sec71p has been shown to induce the UPR (Jonikas et al., 2009). Therefore to investigate whether upregulation of Ysy6p correlated with the level of UPR induction Ysy6p expression levels were investigated in deletion strains with increasing levels of UPR induction as defined by Jonikas et al., (2009). Therefore Ysy6p levels in *Δhrd1, Δder1* and *Δlhs1* strains were also investigated. Hrd1p is the core component of the ERAD-M and ERAD-L pathways whereas Der1p has only been demonstrated to be required in the ERAD-L pathway (Carvalho et al., 2006). Lhs1p is a Hsp70 chaperone involved in protein translocation (Craven et al., 1996, Hamilton and Flynn, 1996). The strains were propagated to log phase is rich YPD media and levels determined by SDS-PAGE and western blotting with anti-Ysy6p antibody followed by reprobing of the nitrocellulose membrane with anti-Zwf1p antibody (figure 5.14a). Signal intensities obtained from western blotting were quantified using Aida image analyser by making boxes of identical size and subtracting background signal. The Ysy6p signal was then normalised to the Zwf1p loading control and the mean and standard error of the mean calculated. Analysis of variance across the *WT*, Δ*sec71*, Δ*der1*, Δ*hrd1*, Δ*lhs1* strains showed no significant difference in Ysy6p levels and therefore that Ysy6p levels could not be correlated with UPR induction. Differences between the *WT* and query deletion strains were tested for significance using an unpaired two tailed t-test. This showed that Ysy6p expression levels between the *WT*

and Δ*sec71* strains were significantly different ($P<0.05$) (Figure 5.14b). This therefore demonstrates that deletion of *SEC71* causes an increase in the expression level of Ysy6p.

5.9 Observing the effect of truncating Ysy6p-opsin on the crosslinking profile.

In chapter 4 it was demonstrated that loss of ribosome association of Ysy6p-opsin was caused by loss of amino acids 7-9 which corresponds to loss of the sequence RQR in truncation T2. It was therefore of interest to investigate whether loss of ribosome association could be correlated with alterations in the crosslinking profile as this might further determine the importance of the cytosolic domain in the function of Ysy6p. Therefore to investigate the effect of truncating Ysy6p-opsin on the crosslinking profile crosslinking was carried out with MBS to a final concentration of 900 μM with membrane extracts prepared from the *BY4742* Δ*ysy6* strain transformed with the Ysy6p-opsin constructs or *pRS315.*

The truncated constructs of Ysy6p-opsin were derived from the Ysy6p-opsin construct containing the glycosylatable opsin tag. Previous crosslinking of Ysy6p-ospin was carried out using the construct which does not contain a glycosylation site (Figure 5.4). The crosslinking profiles of the full length Ysy6p-opsin glycosylatable and unglycosylatable constructs were therefore also compared. As can be seen in figure 5.15, the unglycosylated construct displays three crosslinking products of 32 kDa (A), 28 (B) and 19 kDa (C) as previously observed in Figure 5.15. The full length glycosylatable (Ysy6p-opsinG) construct shows these same bands with 4 additional bands three of which are specific for crosslinking with MBS. A band migrating at approximately 10kDa (1) represents unglycosylated Ysy6p-opsin. Glycosylated Ysy6p-opsin is represented by a band of 16 kDa (2). In addition three other crosslinker treated bands of 34 kDa (A'), 30 kDa (B') and 24 kDa (C'). Bands A', B' and C' are consistent with the shift expected for crosslinking of glycosylated Ysy6p-ospin (te Heesen et al., 1992). Interestingly, the glycosylatable Ysy6p contains additional lysines at the C terminus as shown in figure 4.9. However this did not lead to additional crosslinking bands which could not be attributed to glycosylation of Ysy6p.

Truncating Ysy6p-opsin did not abolish crosslinking. The crosslinks became smaller in size in correlation to extent of truncation. However, crosslinks A, A', B and B' crosslinks were severely diminished in the third truncation corresponding to a loss of the sequence LANAKF in truncation T3. Crosslinks C and C' however were unaffected by truncation of Ysy6p-opsin. Interestingly, all truncations of Ysy6p-opsin caused the appearance of a 46 kDa band. In summary, it has been demonstrated that truncating the cytosolic domain of Ysy6p-opsin causes near loss of crosslinking to two crosslinking partners of approximately 18 and 22 kDa but does not affect crosslinking to a third crosslinking partner of approximately 10 kDa.

5.10 Discussion

Crosslinking of endogenous Ysy6p and Ysy6p-opsin gave rise to three crosslinking products of 32 kDa, 28 kDa and 19 kDa. It has therefore been shown that both endogenous Ysy6p and the Ysy6p-opsin overexpression construct can crosslink to three factors of approximately 22 kDa, 18kDa and 10 kDa. The Ysy6p-opsin construct showed additional weak crosslinks of 29 kDa and 31 kDa when crosslinking was performed at 24°C. These 29kDa and 31kDa bands became more efficiently formed at 37°C. Two reasons can be offered to explain the appearance of these additional Ysy6p-opsin specific crosslinks. Firstly, these crosslinks relate to factors which are inefficiently formed and therefore poorly detectable at endogenous levels of Ysy6p. Secondly, these crosslinks are the result of over-expression of Ysy6p-opsin and may not be representative of factors which are in close association with endogenous Ysy6p. The Ysy6p-opsin therefore gives rise to three physiologically relevant crosslinks of 28 kDa, 32 kDa and 19 kDa when crosslinking is performed with MBS at 24°C. Furthermore these crosslinking factors could be immunoprecipitated using the Ysy6p antibody. Silver staining of immunoprecipitations of Ysy6p and its crosslinking partners gave rise to weak silver stained bands. However, the observed crosslink could not be identified by mass spectrometry. The weakness of silver staining was shown to be due to a low yield of Ysy6p-opsin and not due to inefficiency in silver staining. This was done by comparison of both western blotting and silver staining of the Ysy6p-opsin precipitate and purified RAMP4-opsin. Furthermore, it was shown that the total amount of Ysy6p-opsin immunoprecipitated was of the order of 10 to 100 ng. When the

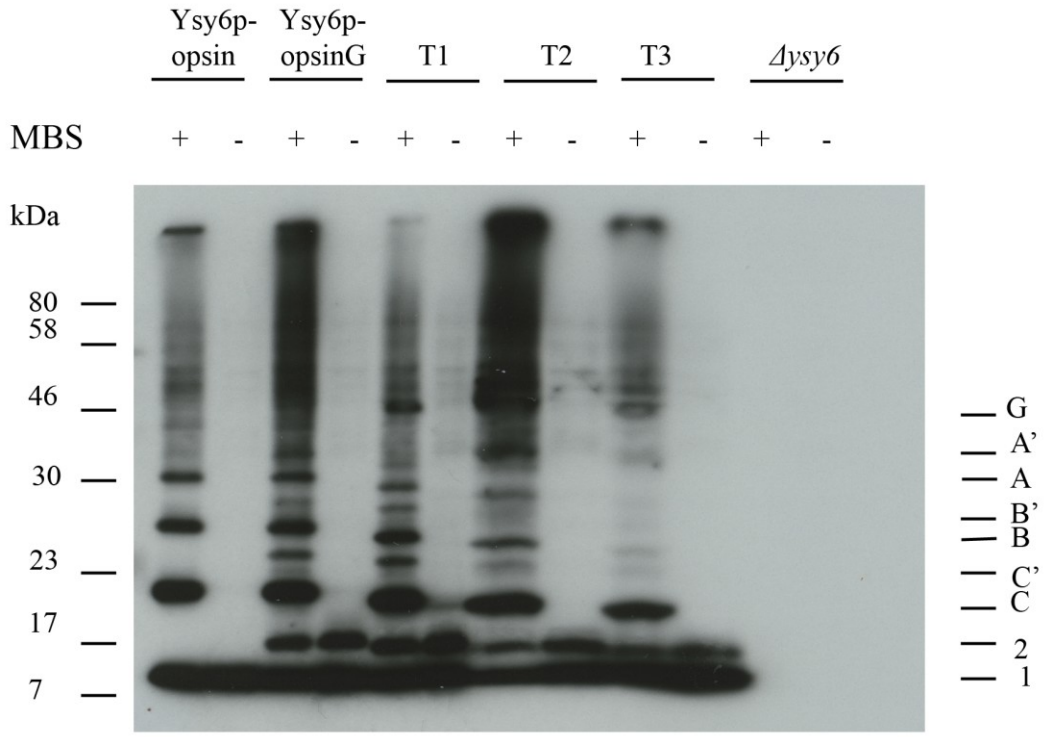

Figure 5.15 Comparing the crosslinking profiles of full length and truncated Ysy6p-opsin. The *BY4742* Δ*ysy6* strain transformed with either *pRS315*, *pRS315-YSY6N, pRS315-YSY6NQ, pRS315-YSY6NQT1, pRS315-YSY6NQT2* or *pRS315-YSY6NQT3* were propagated in SD media lacking leucine and methionine. Membrane preparations were made and treated with 900 μM MBS or mock treated with DMSO and incubated at 24°C for 20 minutes. Samples were loaded onto a 12.5% SDS-PAGE gel and western blotted using the anti opsin antibody. Amount of sample loaded per lane of cell equivalent membrane preparations: 2.5 OD_{600} of Ysy6p-opsin, 2.5 OD_{600} of Ysy6p-opsinG T1, 5 OD_{600} of T2, 7.5 OD_{600} of T3 and 7.5 OD_{600} negative control *Δysy6* strain. Representative western blot of 3 repeats experiments.

crosslinking products were immunoprecipitated they would have only constituted a fraction of this amount as not all Ysy6p-opsin is crosslinked. This suggests that the crosslinking partner could not be identified due to insufficient material being precipitated. However, the yield of Ysy6p-opsin could be potentially further increased by immunoprecipitation using the anti-opsin antibody. The opsin antibody is a purified monoclonal antibody, in contrast to the whole serum Ysy6p antibody, and therefore allows for a greater amount of Ysy6p-opsin specific antibody to be used in the immunprecipitation and subsequent silver staining of the SDS-PAGE gel. It was also demonstrated that the 32 kDa and 28 kDa crosslinks migrate close to antibody light chains and constitutes one reason why these crosslinking partners could not be observed by silver staining. A strategy which could be used to separate these crosslinks from the antibody light chains would consist of crosslinking the Ysy6p anitbody to the protein A sepharose beads prior to immunoprecipitation of the Ysy6p-opsin crosslinks (Harlow and Lane, 1988). The results therefore validate the approach of using the Ysy6p-opsin overexpression construct for crosslinking. In addition, crosslinking with DSS resulted in a strong crosslinking product of 20 kDa the identity of which could also be investigated using a similar approach.

The mammalian homolog of Ysy6p, RAMP4, is a ribosome associated protein which has been shown to be involved in protein integration and affects the degradation of proteins (Pool, 2009, Schroder et al., 1999, Yamaguchi et al., 1999, Görlich and Rapoport, 1993). In addition, as demonstrated in chapter 4, Ysy6p is a ribosome associated protein and it was also demonstrated in chapter 3 that deletion of *YSY6* affects ERAD. Therefore in an effort to identify the 22 kDa and 18kDa crosslinking factors a screening approach was used to investigate potential loss of crosslinking in deletion strains for genes of candidate proteins involved in processes associated with the function of RAMP4 and Ysy6p such as protein integration and ERAD. However crosslinking in deletion strains did not lead to any observation of loss of crosslinking. Therefore it has been excluded that Ysy6p crosslinks to Sec71p, Sec72p, Cue1p and Ubc7p. However, a more detailed search of candidate crosslinking partners yielded additional candidate croslinking partners. It has been shown in chapter 4 Ysy6p is a ribosome associated protein and therefore it is possible that Ysy6p crosslinks to ribosomal proteins. Supporting this hypothesis is the fact that the mammalian homolog of RAMP4 has been shown to crosslink to the ribosomal protein rpl17 (Pool, 2009). In

order to determine candidate ribosomal proteins which could potentially crosslink to Ysy6p the structure of the yeast ribosomal 60S subunit in complex with the Sec61p homolog, Ssh1p were aligned as previously determined by Becker et al., (2009). It was determined in chapter 4 that the cytosolic domain of Ysy6p consists of the first 43 amino acids which is followed by the transmembrane domain. The length of this cytosolic domain was estimated to be of approximately 55-60Å on the basis of the cytosolic domain forming a linear alpha helix. To this length the length of the arm length of MBS was taken into account. The spatial constraints for crosslinking of Ysy6p with ribosomal proteins were therefore estimated to be of 65Å from the ER membrane (Figure 5.16). Ribosomal proteins in agreement with this distance constraint were then screened for cysteine content and agreement with the size of the crosslinking products. This yielded three proteins, namely, Rpl43p, Rpl37p and Rpl35p of 10 kDa, 10kDa and 14kDa respectively (Figure 5.16). These candidate crosslinking partners are consistent with the size of the 20kDa crosslink. No candidates could be identified for the 28kDa and 32 kDa crosslinking products.

In addition to ribosomal proteins candidate crosslinking partners were more extensively screened in processes associated with protein integration and ERAD to include the signal sequence processing, protein glycosylation and ERAD-L. The additional partners identified include Spc1p, Ost2p, Ost5p and Der1p. Spc1 is a 10kDa subunit of the signal peptidase complex (Fang et al., 1996). Ost2p and Ost5p are respectively 15 kDa and 10 kDa members of the OST complex which mediates glycosylation of proteins (Reiss et al., 1997, Silberstein et al., 1995). Evidence of the involvement of Ysy6p in glycosylation has been previously observed for the mammalian homolog of Ysy6p, RAMP4 by the fact that RAMP4 has been shown facilitate glycosylation of proteins (Yamaguchi et al., 1999). Lastly, Der1p is a 24kDa protein involved in ERAD-L (Hitt and Wolf, 2004). Intrestingly, Ysy6p shows a genetic interaction with *DER1* and with *WBP1* which constitute another member of the OST complex (Costanzo et al., 2010, Schuldiner et al., 2005). Deletion strains of *SPC1*, *OST5* and *DER1* are viable and therefore crosslinking could be carried out in deletion strains for these genes to investigate loss of crosslinking (Giaever et al., 2002). However, deletion of ribosomal proteins and *OST2* are inviable and therefore investigation of its ability to crosslink Ysy6p would have to be carried out using a comparative western blotting approach using an anti Ost2p and opsin antibody (Giaever et al., 2002).

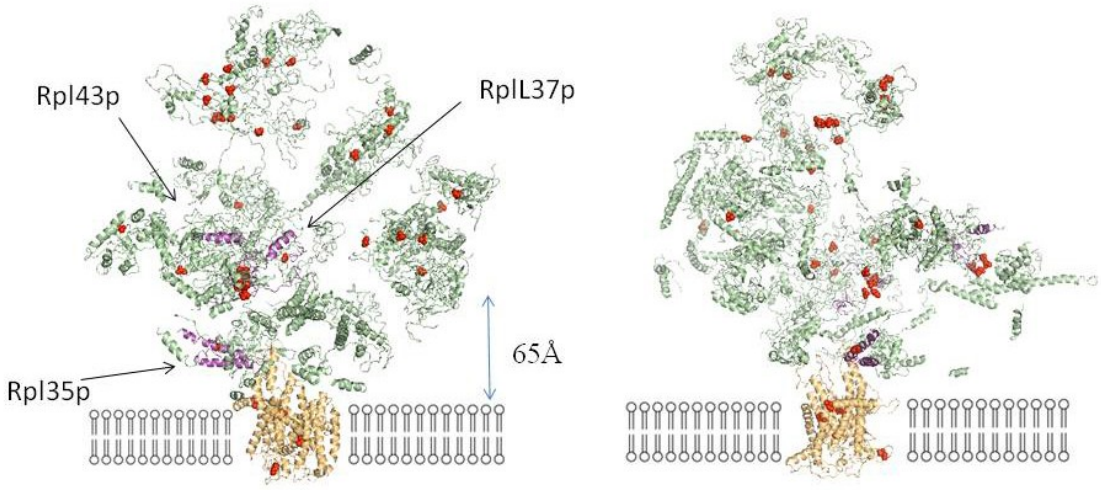

Figure 5.16 Candidate ribosomal proteins involved in crosslinking to Ysy6p-opsin. Alignment of the Cryo EM structure of Ssh1p (gold) complex with the crystal structure of the yeast 60s ribosome subunit (green) using PyMol (PDB codes: 2WW9 and 3O58). The Sec61p homolog, Ssh1p, was placed in the membrane as previously reported (Becker et al., 2009). A length of 65 Å was then measured from the membrane and used as a length contstraint for candidate proteins of interest. Cysteine residues (red) were identified in ribosomal proteins (green). Cysteine containing ribosomal proteins of sizes ranging from 9 kDa to 30 kDa in agreement with the length constraint of 65 Å were then identified (magenta).

Sec71p has been shown not to crosslink to Ysy6p with MBS. However, *YSY6* and *SEC71* have been shown to genetically interact due to the fact that deletion of *SEC71* results in upregulation of Ysy6p. Deletion of *SEC71* causes induction of the UPR. However, other deletion strains investigated with reported upregulation of the UPR did lead to upregulation of Ysy6p. This suggests that Ysy6p is not upregulated by the UPR which is consistent with previous findings that Ysy6p is not a UPR target (Travers et al., 2000). Sec71p is a member of the Sec62/Sec63 complex involved in both cotranslational and post-translational protein import (Jermy et al., 2006, Young et al., 2001). Interestingly, Sec62p has been shown to have a physical interaction with Ysy6p by two hybrid screening (Yu et al., 2008). Furthermore, it has been shown that deletion of *YSY6* and *SEC71* gives rise to a weaker than predicted induction of the UPR and therefore that double deletion of *YSY6* and *SEC71* has an alleviating genetic interaction (Jonikas et al., 2009). Alleviating interactions between two genes are suggestive of the function of the genes being involved in a shared biochemical pathway (Jonikas et al., 2009). Together the data therefore suggest that Ysy6p is involved with protein integration processes mediated by the Sec62/Sec63 complex.

Having identified that Ysy6p-opsin crosslinks to multiple proteins it was investigated whether crosslinking was dependent on the presence of the N terminal cytosolic sequence of Ysy6p-opsin. It was demonstrated that deletion of amino acids 3 to 6 (T1), 3 to 9 (T2) or 3 to 15 (T3) resulted in the formations of an additional crosslink of 46 kDa. This therefore shows that loss of the N terminal sequence causes a change in the molecular environment of Ysy6p-opsin. The previously observed 32 kDa, 28 kDa and 19 kDa crosslinks persisted across all truncations. However, the 32 kDa crosslink and the 28 kDa crosslink were nearly abolished in truncation T3 which demonstrates that amino acids 10 to 15 are required for efficient crosslinking. Truncation T3 contains the first lysine residue of Ysy6p-opsin at position 14. Therefore the reduction in formation of the 32 kDa and 28 kDa crosslinks may be attributable to the deletion of lys14. Notably, however the 32 kda and 28 kDa crosslinks are severely reduced in intensity but not abolished which suggests that either amino acids 10 to 15 promote interaction with Ysy6p-opsin and the crosslinking partner or alternatively multiple lysines of Ysy6p-opsin are able to generate the 32 kDa and 28 kDa crosslinking products. This alternative crosslinking site could be provided by the following lysine at position 17 which is not deleted in truncation T3. The data therefore demonstrate that the 32 kDa

and 28 kDa crosslinks are formed primarily due to a close spatial proximity between lys14 and a cysteine residue in the crosslinking partner and therefore that the N terminal domain is in close proximity to other proteins. The formation of the 19 kDa crosslink is however not dependent on the presence of amino acids 3 to 15. This suggests that both crosslinking and interaction sites between Ysy6p and 10 kDa crosslinking partner are dependent on downstream sequences from amino acid 15 of Ysy6p-opsin.

In summary, crosslinking of Ysy6p-opsin overexpression gives rise to physiologically relevant crosslinking products which can be immunoprecipitated. The Ysy6p antibody was shown to only precipitate limited amounts of Ysy6p-opsin. There are however promising alternative approaches which can be used for identification of these crosslinking products. The number of candidate crosslinking partners to Ysy6p-opsin has been narrowed down on the basis of localisation, size, cysteine content and implication in processes already implicated in the function of Ysy6p and RAMP4 to seven candidate proteins namely; Spc1p, Ost2p, Ost5p, Der1p, Rpl43p, Rpl37p, and Rpl35p. The involvement of these proteins in crosslinking to Ysy6p can therefore be investigated using a comparative western blotting approach by either comparing deletion strains to the *WT* for loss of crosslinking or by using antibodies for each of the candidate proteins. It can however not be excluded that Ysy6p crosslinks to other factors which may be related to other factors involved in other unidentified processes. In this case there are still good perspectives for identification of the crosslinks as immunoprecipitation with the anti opsin antibody may be performed. This strategy would also permit identification of the 10 kDa crosslinking partner observed when crosslinking was performed with DSS.

Sequential deletion of the N terminus of Ysy6p-opsin has shown that amino acids 3 to 10 are not required for crosslinking with MBS. However, amino acids 10 to 15 are required for efficient crosslinking to two factors of approximately 20kDa. A third MBS formed crosslinking partner of approximately 10 kDa however was shown not to be affected by deletion of amino acids 10 to 15. The data therefore suggest that distinct crosslinking partners require distinct domains of Ysy6p for crosslinking. Furthermore, considering the abundance of lysines in Ysy6p the data suggest that the N terminal

domain is required for a close interaction with two proteins of approximately 20 kDa which permit crosslinking.

CHAPTER 6: Concluding remarks

The first objective of this study was to determine whether Ysy6p is a functional homolog of RAMP4. The most striking evidence that Ysy6p is a functional homolog of RAMP4 has been demonstrated by the fact that Ysy6p similarly to RAMP4 can form a tight association with ribosomes (Görlich and Rapoport, 1993). Ribosome association of RAMP4 implicated its function in protein integration. This was later supported by observations that: RAMP4 improves integration and glycosylation of membrane proteins; is recruited to ribosomes specifically during the emergence of transmembrane domains in the ribosomal exit tunnel; and that RAMP4 can be crosslinked to nascent proteins (Pool, 2009, Hori et al., 2006, Schroder et al., 1999, Yamaguchi et al., 1999). However, RAMP4 has not been shown to be required for protein translocation. Similarly, deletion of *YSY6* did not lead to any observation of defects in protein integration for both co-translationally and post-translationally integrated or translocated substrates. However, it was observed that deletion of *SEC71* causes increased expression of Ysy6p and therefore that *YSY6* genetically interacts with *SEC71*. It can therefore be hypothesised that Ysy6p levels are upregulated to compensate for the loss of function of Sec71p. Previously reported data supports this hypothesis. Firstly, Sec62p has been reported to physically interact with Ysy6p (Yu et al., 2008). Secondly that the relative induction of the UPR in the double deletion strain of *SEC71* and *YSY6* is lower than the expected level determined in single deletion strains for *YSY6* and *SEC71* (Jonikas et al., 2009). This so called alleviation of UPR induction suggests that Ysy6p and Sec71p operate in a linear pathway (Jonikas et al., 2009). Therefore evidence is mounting that Ysy6p is not only ribosome associated but also functions in integration processes associated with the Sec62/Sec63 complex (Jermy et al., 2006, Willer et al., 2003, Young et al., 2001, Green et al., 1992). Ysy6p and RAMP4 are therefore both in tight association with ribosomes and there are multiple lines of evidence that both proteins function in protein integration.

Deletion of *YSY6* has been shown to affect the degradation of membrane proteins by causing a transient stabilisation of the ERAD-M degradation substrate Hmg2-6myc which is followed by rapid degradation (Hampton et al., 1996). Overexpression of RAMP4 has been shown to have a protective effect on the degradation of the membrane proteins RAGE and CD8 (Yamaguchi et al., 1999). Therefore the function

of both RAMP4 and Ysy6p have been shown to be implicated in protein degradation. However, Yamaguchi et al., (1999) showed that it is the over-expression of RAMP4 which has a protective effect on the degradation of proteins whereas this study suggests that it is the deletion of *YSY6* which leads to a transient protective effect on the degradation of Hmg2-6myc. Therefore the question is how could both deletion and overexpression of RAMP4 and Ysy6p have a protective effect on the degradation of proteins? This conundrum can be explained by two alternative models where Ysy6p and RAMP4 have a regulatory role in the degradation of proteins. The mammalian homolog of Ysy6p, RAMP4, has been shown to associate with hydrophobic sequences of proteins during co-translational translocation of proteins and be in tight association with the Sec61p complex even when ribosomes are removed (Schroder et al., 1999, Görlich and Rapoport, 1993). This suggests that RAMP4 interacts with nascent proteins and Sec61p via hydrophobic interactions. Strikingly, the substrate used by Schroder et al., (1999) shows association with RAMP4 in a manner which is concomitant with exit of the N terminal transmembrane domain from the translocon and the presence of a hydrophobic sequence inside the translocon. In addition, it is known that unstable or poorly hydrophobic domains sometimes need to reassociate with a previously synthesised transmembrane domain in order to exit the translocon (Rapoport et al., 2004, Heinrich and Rapoport, 2003, Skach and Lingappa, 1993, Lin and Addison, 1995). Recruitment of RAMP4 coincides with the presence of a hydrophobic domain in the translocon and the presence of a previously synthesised transmembrane domain in the ER (Schroder et al., 1999, Devaraneni et al., 2011). This hydrophobic sequence of Ii is normally translocated into the ER lumen. However, it can therefore be hypothesised that the function of RAMP4 is to shield the two hydrophobic domains from each other and preventing erroneous partitioning of a hydrophobic sequence into the lipid bilayer due to reassociation with a previously synthesised transmembrane domain. Furthermore, overexpression of RAMP4 could lead to a prolonged association with protein substrates thereby shielding them from degradative processes (Yamaguchi et al., 1999). This therefore suggests a regulatory role for RAMP4 in ERAD. Deletion of Ysy6p on the hand may increase the abundance of proteins with aberrantly associated transmembrane domains which may be inefficiently recognised by the ERAD machinery and promotes the induction of alternative pathways for degradation. However, a second model for the function of RAMP4 can be put forward. It is also possible that prolonged association of substrate proteins with Ysy6p causes the

recruitment of factors which cause degradation of substrates. RAMP4 was shown to have a protective effect on proteins only after induction of the UPR whereas deletion of *YSY6* was shown to promote degradation only after incubation with cycloheximide which is known to induce the UPR (Shenkman et al., 2007, Yamaguchi et al., 1999). It is therefore possible that Ysy6p in fact protects proteins from degradation under conditions of ER stress thus prolonging the time allowed for proteins can achieve a native state. However, loss of *YSY6* was also shown in this study to cause a transient stabilisation of Hmg2-6myc. This transient stabilisation can however only be explained if Ysy6p has a function in promoting ERAD. Therefore the data suggest that Ysy6p has function in recruiting ERAD factors and hence Ysy6p must have a regulatory role in ERAD. The manner in which Ysy6p affects degradation of proteins is therefore consistent with that of RAMP4 and therefore further indicates that Ysy6p is a functional homolog of RAMP4. Together the data is consistent with the notion that Ysy6p is a functional homolog of RAMP4 which functions in protein integration and ERAD.

In order to obtain an insight into the sequence requirements for a tight association of Ysy6p with ribosomes deletions of segments of the cytosolic domain of opsin tagged Ysy6p were performed. This showed that deletion of amino acids 3 to 7 of Ysy6p did not affect ribosome association. However, further deletion of downstream amino acids lead to a near loss of ribosome association. Loss of ribosome association was shown to relate to deletion of an N terminal segment of Ysy6p predicted to form part of greater conserved sequence which forms a helix-turn-helix secondary structure.

When membrane preparations were treated with MBS Ysy6p and Ysy6p-opsin formed three major crosslinking products of 32 kDa, 28 kDa and 19 kDa. Therefore, both Ysy6p and Ysy6p-opsin crosslink to two factors of approximately 20 kDa and an additional factor of approximately 10 kDa. The fact that the formation of these crosslinks could be observed for both Ysy6p and Ysy6p-opsin suggests that the molecular associations of both proteins are similar and that both overexpression and tagging of Ysy6p-opsin does not ostensibly alter the function of Ysy6p. To further investigate the effect of deleting segments of the cytosolic domnain of Ysy6p crosslinking of full length and truncated Ysy6p-opsin crosslinking with MBS was performed. This showed that crosslinking to the 10 kDa factor is unaffected by deletions in the cytosolic domain of amino acids 3 to 15. Strikingly, crosslinking to two

factors of 19 kDa was lost when the first helical segment of the conserved helix-turn-helix motif was completely deleted. The turn region contains the only two strictly conserved amino acids, Lys17 and Asn18. This suggests that loss of crosslinking to the 19 kDa factors is attributable to disruption of secondary structure which maintains these two residues in a conformation which permits interaction with crosslinking partners. Alternatively, due to the fact that loss of crosslinking is concomitant with deletion of Lys14 it is also possible that crosslinking is lost because Lys14 constitutes the primary site for crosslinking to the 19 kDa factors. However, Ysy6p contains numerous lysines throughout its cytosolic domain and therefore contains multiple opportunities for crosslinking. This suggests that Lys14 is in close proximity with its crosslinking partner and therefore also suggests that the helix-turn-helix motif is important for the interaction of Ysy6p and its crosslinking partner.

A near loss of Ribosome association of Ysy6p is observed when only part of the first conserved alpha helical domain is lost whereas crosslinking was shown to persist. This discrepancy can be explained by the fact that ribosome association was investigated under conditions of high salt and presence of detergent whereas crosslinking was performed under conditions of low salt in intact membranes. Therefore the data suggest that the helix-turn-helix domain is required for maintaining Ysy6p in a conformation which allows for a salt resistant association with ribosomes. However, under conditions of low salt and absence of detergent partial deletion of the first conserved helical domain is not sufficient to disrupt molecular interactions. This comparison between data from ribosome sedimentation and crosslinking has assumed that the crosslinking products formed relate to factors involved in mediating ribosome association. Considering that Ysy6p forms a tight association with ribosomes it can be hypothesised that ribosomal proteins or other ribosome associated proteins constitute likely candidates for crosslinking. However, since the function of Ysy6p has now been extended to a poorly characterised role in ERAD it cannot be excluded that factors which crosslink to Ysy6p represent interactions to a second pool of Ysy6p which is not ribosome associated. Future identification of factors which crosslink to Ysy6p would therefore shed light on this and potentially further rationalise the role of Ysy6p in ERAD. It is however clear that an helix-turn-helix motif plays an important role in the molecular associations of Ysy6p.

The overall aim of this project was to investigate candidate factors for involvement in processes relating to ER function. The first objective of this study was to determine the functional similarities between Ysy6p and RAMP4 as a way of investigating how the function of Ysy6p related to ER processes. The second objective of this study was to obtain an insight into the function of the EMC complex which has been suggested to function in protein integration and ERAD and shows genetic interactions with Ysy6p. It has previously been shown that in the presence of the UPR inducing agent, tunicamycin, *Δemc1*, *Δemc3* and *Δemc5* complex members becomes lethal in the absence of *HAC1* (Bircham et al., 2011). In addition, it has been shown that members of both the mammalian and yeast EMC complex are upregulated by the UPR (Christianson et al., 2012, Travers et al., 2000). Therefore there is pre-existing evidence that the *EMC* complex is involved in the UPR. However, the *EMC* complex has only been shown to be required when the UPR is compromised due to deletion of *HAC1* (Bircham et al., 2011). In addition, deletions of the EMC complex members and *HAC1* are only lethal in the presence of tunicamycin and not DTT and therefore that the *EMC* complex is not ubiquitously required when the UPR is compromised. In this work it has been demonstrated that single deletions of EMC complex members *EMC1*, *EMC2*, *EMC3*, *EMC6* leads to severe competitive growth defects under general stress inducing conditions caused by both elevated temperature and exposure to SDS. Therefore it has been demonstrated that the *EMC* complex is directly involved in stress responses.

Increased sensitivity to SDS is an indication of defective cell wall or membrane biogenesis which in turn implicates defects in processes related to protein biogenesesis (Bickle et al., 1998). Therefore in order to further investigate whether SDS sensitivity was due to factors affecting protein biogenesis specific pathways were tested. Involvement of the EMC complex in protein integration and translocation using both co-translational and post-translational substrates were tested but no defect in protein integration could be observed. It has previously been shown that deletion of members of the *EMC* complex gives rise to ER retention of the substrate Mrh1p-GFP (Bircham et al., 2011). However, no alteration in trafficking of the substrate Yor1p-GFP could be detected when *EMC5* is deleted. The reasons why Mrh1p-GFP is retained in the ER is unclear. However, the fact that Yor1p-GFP trafficking is unaltered in the Δ*emc5* strain suggested that retention of Mrh1p-GFP is not due to a generalised defect in forward trafficking from the ER.

Strikingly, however deletion of *EMC5* has been shown to cause a defect in ERAD-M due to defective clearance of Hmg2-6myc. This finding is consistent with reports that the mammalian EMC complex forms part of the ERAD network (Christianson et al., 2012). It is however noteworthy that deletion of *EMC1* does not lead to a defect in ERAD and deletion of *EMC5* does not lead to a substantial change in competitive fitness at either elevated temperature or in media containing SDS whereas deletion of *EMC1* has a pronounced effect. This suggests that even though the EMC is a stoichiometric complex individual members possess distinct functions which mediate or coordinate multiple processes. How this is possible is unclear and clearly requires more investigation but already suggests that ERAD and stress responses are mediated by additional level of complexity than previously characterised.

In summary it has been demonstrated that the function of Ysy6p is consistent with the function of RAMP4 and therefore that the two proteins are functional homologs involved in ERAD and protein integration. It has also been demonstrated that a segment ofa cytosloic helix-turn-helix motif of Ysy6p is required for efficient ribosome association. In addition, it has been demonstrated that the EMC complex is involved in stress responses and ERAD.

CHAPTER 7: References

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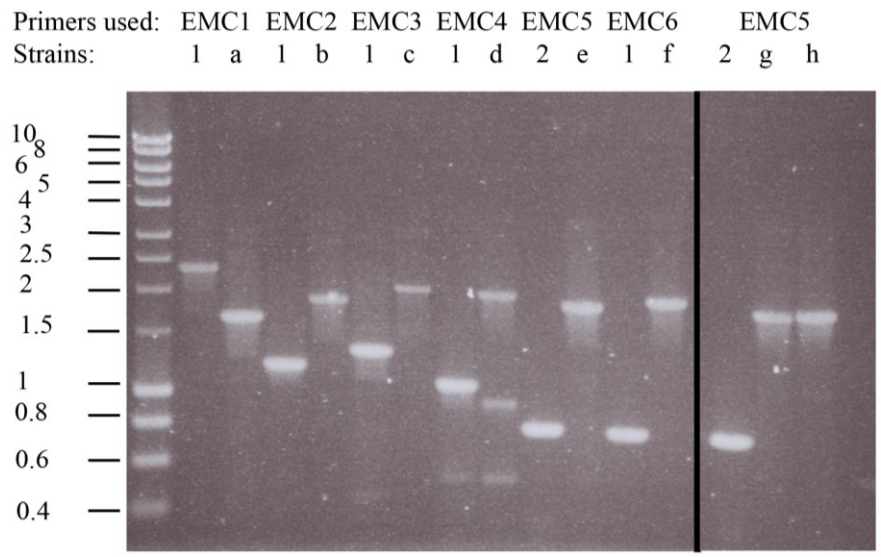
APPENDIX I

a. Confirmation of strain identity using screening primers.



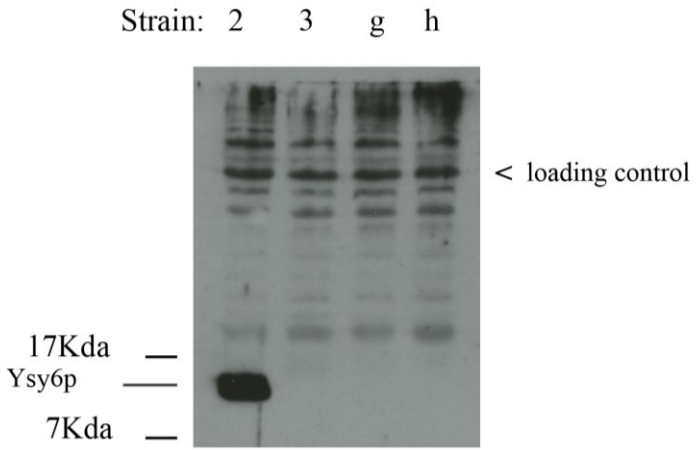
Expected band sizes (bp):

EMC1	2373	*EMC4*	1092
Δ*emc1*	1692	Δ*emc4*	2020
EMC2	1219	*EMC5*	809
Δ*emc2*	1941	Δ*emc5*	1984
EMC3	1347	*EMC6*	792
Δ*emc3*	2186	Δ*emc6*	2066

Strains:

1: *WT BY4741*
2: *WT BY4742*
a: Δ*emc1 BY4741*
b: Δ*emc2 BY4741*
c: Δ*emc3 BY4741*
d: Δ*emc4 BY4741*
e Δ*emc5 BY4742*
f Δ*emc6 BY4741*
g. Δ*emc5/* Δ*ysy6* (a)
h. Δ*emc5/* Δ*ysy6* (b)
3. Δ*ysy6 BY4742*

b. Confirmation of deletion of *YSY6* by blotting



Appendix I Confirmatory PCR and western blotting of deletion strains. a. PCR carried out using screening primers for the gene loci. PCR product sizes from the *WT* locus was compared to fragment sizes after replacement of the gene with the 1.6 kpb KanMX cassette. **b**. SDS-PAGE of 0.3 OD_{600} units of cell culture equivalent of total cell extract followed by western blotting with Ysy6p antibody. The cross-reacting band (<) used as a loading control

APPENDIX II

T1 T2 T3

YSY6: ATGGCCGTACAGACACCAAGACAAAGACTGGCTAATGCCAAGTTTAA
CAAGAATAACGAAAAGTATAGAAAATACGGTAAGAAGAAGGAGGGCA
AAACTGAGAAAACCGCACCTGTGATATCCAAAACTTGGTTGGGTATTC
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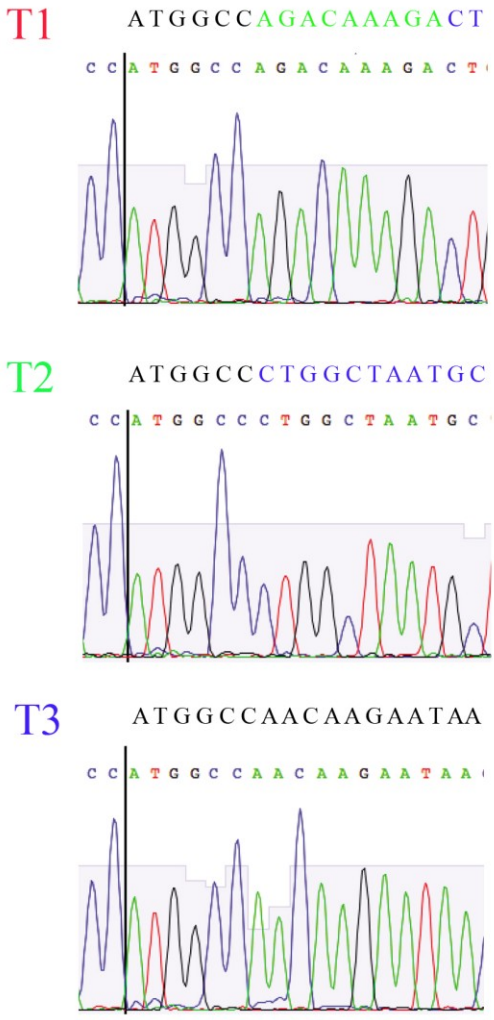
CTATGA



Appendix II. Sequencing results of truncated products T1, T2, T3. Sequencing carried out using reverse primer to the Ysy6p open reading frames. Deleted sections are shown in red, green and blue for T1 (red), T2 (red and green) and T3 (red, green and blue).