Lipid perturbation compromises UPR-mediated ER homeostasis

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Abbreviations:

ACT	Artemisinin Based combination therapy
BiP	Binding immunoglobulin protein
cDNA	Complementary DNA
CHX	Cycloheximide
CL	Cardiolipin
Co-IP	Co-immunoprecipitation
CTP	Cytidine-5'-triphosphate
CPY	Carboxypeptidase Y
CVD	Cardiovascular disease
DAG	Diacylglycerol
DMPE	N,N-dimethyl phosphatidylethanolamine
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DOPC	Dioleoyl-phosphatidylcholine
DOPE	Dioleoyl-phosphatidylethanolamine
DPPC	Dipalmitoylphosphatidylcholine
DPPE	1,2-Bis(diphenylphosphino)ethane
DTBP	Dimethyl 3,3'-dithiobispropionimidate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EMC	ER transmembrane complex
ER	Endoplasmic reticulum
ERAD	Endoplasmic Reticulum-associated degradation
ERAD-C	Endoplasmic Reticulum-associated degradation-Cytosol
ERAD-L	Endoplasmic Reticulum-associated degradation-Lumen
ERAD-M	Endoplasmic Reticulum-associated degradation-Membrane
FRAP	Fluorescence recovery after photobleaching
gDNA	genomic DNA

GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
h	Hour
HFD	High fat diet
HRP	Horseradish peroxidase
HSP	Heat shock protein
HSR	Heat shock response
IP	Immunoprecipitation
kDa	Kilo Dalton
LMD	Lipid metabolism disorders
LP	Lipid perturbation
MD	Molecular dynamics
MLS	Mitochondria localizing signal
MMPE	N-monomethyl phosphatidylethanolamine
mRNA	Messenger ribonucleic acid
NAE	N-acylethanolamine
NAFLD	Non-alcoholic fatty liver disease
OE	Overexpressing
O	Origin
OD	Optical density
OST	Oligosaccharyl transferase complex
PBS	Phosphate buffer saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
PG	Phosphahtidylglycerol
PIC	Protease inhibitor cocktail
PMSF	Phenylmethylsulfonyl fluoride

qPCR	Quantitative polymerase chain reaction
RIDD	Regulated Ire1-dependent decay
RT	Room temperature
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SC	Synthetic complete
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SERCA	Sarcoplasmic reticulum Ca ²⁺ -ATPase
SGD	<i>Saccharomyces</i> Genome Database
SRP	Signal recognition particle
T2D	Type II diabetes
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
Tm	Tunicamycin
TOM	Transporter outer membrane
TP	Transmembrane protein
UPR	Unfolded protein response
WHO	World Health Organization
WT	Wild type
YPD	Yeast peptone dextrose

Publications

Wu, H.*, **Ng, B. S.***, & Thibault, G. (2014). Endoplasmic reticulum stress response in yeast and humans. *Biosci Rep.* (*co-first authors)

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Summary

Phospholipid homeostasis in biological membranes is essential to maintain cellular functions of organelles such as the endoplasmic reticulum (ER). Obesity has been associated to the perturbation of the ratio of the two most abundant phospholipids in the ER, phosphatidylcholine (PC) and phosphatidylethanolamine (PE). PC/PE disequilibrium has also been linked to metabolic diseases such as non-alcoholic fatty liver disease and type II diabetes, but its biological significance remains unclear. This study aims to develop a model of how lipid perturbation (LP) can develop into diseased states. Previously, we reported that *Saccharomyces cerevisiae* adapts to lipid disequilibrium by upregulating several protein quality control (PQC) pathways such as the endoplasmic reticulum-associated degradation (ERAD) pathway and the unfolded protein response (UPR). We investigated the PQC pathways at their proteomic levels to investigate their effectiveness in response to LP. Surprisingly, we observed certain ER-resident transmembrane proteins (TPs), part of the UPR programme, to be destabilised under LP. Localisation and integration of the TPs to the ER remain unaffected, suggesting that the TPs might be destabilised by changes in ER membrane composition. The ER membrane was found to have undergone fatty acid remodelling and membrane stiffening. Among these, Sbh1, a member of the translocation complex, was prematurely degraded by dissociating from the Sec61 complex. Sbh1 is targeted for degradation through its highly conserved lysine residue near the membrane in a Doa10-dependent ERAD manner. Further investigations revealed Sbh1 destabilisation could result in the translocational defect under LP. Sbh1 overexpression fails to fix the translocational defect, but ameliorate the protein processing defects found under LP. This suggests a novel role of Sbh1 in protein processing. In addition, as LP compromises the UPR, disrupting phospholipid homeostasis may be exploited to target pathogens that upregulate the UPR for survival. Two lipid genes, *PfFMP* and *PfPSD* were characterised in *Plasmodium*

falciparum by genetic complementation in yeast. Premature removal of key ER-resident TPs under prolonged LP might be an underlying cause of chronic ER stress in metabolic disorders.

Chapter 1. Introduction

1.1. Lipid homeostasis is essential in biological systems

Lipids are one of the major constituent of our body. They consist of a large diverse group of water-insoluble molecules that include fatty acids, glycerolipids, phospholipids, sphingolipids as well as sterol lipids. Fatty acids are the major building blocks of structurally complex lipids such as triacylglycerides. Triacylglycerides, a type of glycerolipids, are mainly used for energy storage and comprise of the bulk of fat storage in animal tissues. Phospholipids, sphingolipids and sterol lipids contribute to the major structure of biological membranes. Several sphingolipids and sterol lipids have also important roles in signalling, functioning as second messengers and as hormones.

Homeostasis, the self-regulating process by which biological systems tend to maintain stability while adjusting to external interferences is essential for survival. By achieving dynamic equilibrium, external forces can continue to exert their influences on the system while a relatively constant internal environment is maintained. Sophisticated homeostasis regulatory mechanisms are employed to regulate lipids (Unger, Clark, Scherer, & Orci, 2010). When present in low amount, lipids are obtained from dietary means or synthesised in the liver. Phospholipids as well as sterol lipids are significantly synthesised and regulated by the liver. When abundant, they are catabolized by the liver for energy or stored in lipid-rich adipose tissues. The maintenance of the relatively constant levels of the diverse lipid species is essential as lipid dysregulation could lead to interference in their functions. This could eventually lead to cellular disruption and to the demise of the cell.

1.2. A primary concern in lipid metabolism disorders is obesity

Disruption in lipid homeostasis has been known to result in lipid metabolism disorders (LMD) (Holthuis & Menon, 2014). LMD could arise from genetic mutations, or by dietary factors such as the excess intake of dietary lipids. A significant number of LMD linked illnesses are caused by genetic mutations (Teslovich et al., 2010) such as Fabry's disease, Gaucher's disease, Niemann-Pick disease, Tay–Sachs disease and familial hypercholesterolemia. However, dietary lipids overload contributes predominantly to LMDs (Parhofer, 2016).

A rising epidemic trend in obesity worldwide especially in developed countries contributes to the rising need to understand the development of the onset of LMDs (Figure 1.1). The prevalence of obesity, which is defined as having a high Body Mass Index (BMI) of 30 kg/m² and above has risen from 3.2% in 1975 to 10.8% in 2014 in men, and from 6.4% to 14.9% in women (Collaboration, 2016). Obesity is a strong risk factor for LMD, and is also linked to a whole myriad of metabolic diseases such as non-alcoholic fatty liver disease (NAFLD), type II diabetes (T2D), cardiovascular diseases (CVD), and cerebrovascular diseases (Ahmed, 2015; Kahn, Hull, & Utzschneider, 2006; Kang, Kim, & Lee, 2017; Lee et al., 2007). Alarming, CVD, cerebrovascular diseases and T2D are the first, second and sixth top causes of deaths globally in 2015, respectively (WHO website, 2015). As obesity is predicted to increase (Finkelstein et al., 2012), obesity related metabolic diseases represent the greatest global public health problem in need for novel solutions (Arruda & Hotamisligil, 2015; Leamy, Egnatchik, & Young, 2013).

While obesity is highly linked to metabolic diseases, the progression from obesity to the pathological conditions remains relatively unclear. Recently, obesity has been shown to lead to a decrease in insulin sensitivity through lipid metabolism changes

(Singla, Bardoloi, & Parkash, 2010). Adiponectin is a hormone important in the regulation of glucose and fatty acid oxidation (S. Li, Shin, Ding, & van Dam, 2009). There is reduced production and sensitivity of adiponectin in obesity, resulting in decreased insulin sensitivity (Haluzik, Parizkova, & Haluzik, 2004; Weyer et al., 2001). Another prominent insight came from evidences that revealed an elevated phosphatidylcholine/phosphatidylethanolamine (PC/PE) ratio in the liver of obese mice (Fu et al., 2011). The perturbation of PC/PE was identified to result in chronic ER stress that leads to the development of NAFLD in the liver. Mice depleted of PC by genetic and dietary means in two different studies were found to develop NAFLD within three days (Z. Li & Vance, 2008; Walkey, Yu, Agellon, & Vance, 1998), which was reversible upon the reintroduction of PC in their diet (Jacobs et al., 2010; Waite, Cabilio, & Vance, 2002). This highlights that obesity can result in phospholipid perturbation that might develop into adverse metabolic diseases.

1.3. Phospholipid homeostasis is highly regulated in biological membranes

Biological membranes serve to generate an internal environment different from cells' exterior, and allow for the compartmentalization of space within the cells themselves. The formation of distinct intracellular environments in organelles allow for metabolic reactions, storage of materials, signalling, as well as sequestration of metabolites.

Phospholipids are a major class of lipids found in biological membranes. Phospholipids are subdivided according to their head group structures, acyl chain lengths and saturation status (Wenk, 2005). They are regulated tightly with significantly different compositions in biological membranes of organelles (Figure 1.2) (van Meer, Voelker, & Feigenson, 2008).

Figure 1.1. Age-standardized mean BMI in men and women by country in 2014.

High average body-mass index (BMI) is prevalent worldwide in (A) men and (B) women. Adapted from (Collaboration, 2016).

Figure 1.2. Lipid composition of biological membrane in *Saccharomyces cerevisiae*.

Lipid composition of plasma membrane, endoplasmic reticulum, mitochondria and vacuole was separated by thin-layer chromatography and obtained by gas-liquid chromatography. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CL, cardiolipin; PA, phosphatidic acid. Adapted from (Zinser et al., 1991).

Phospholipid composition and synthesis are similar in yeast and mammalian cells. However, yeast cell has a simpler fatty acid composition as compared to higher eukaryotes, which promote membrane lipid analysis studies (Daum, Lees, Bard, & Dickson, 1998). Some differences exist such as the biosynthesis of phosphatidylserine, which requires CDP-diacylglycerol in yeast but not in higher eukaryotes. Yeast can also tolerate huge variations in membrane lipid composition, making it ideal for investigating underlying principles and mechanisms which regulates membrane integrity and function (de Kroon et al., 2013).

Phosphatidylcholine (PC) is a major component of biological membranes. Having a cylindrical shape with a cross-sectional area for the head group similar to the acyl chain tails, PC generates minimal curvature and is suitable in forming flat lamellar phase phospholipid bilayer (Szule, Fuller, & Rand, 2002). Phosphatidylethanolamine (PE) is another abundant phospholipid in biological membranes. Having a cone-shaped cross-sectional area where the head group is much smaller than the acyl chain tails, it is reported to form non-lamellar membrane structure and generates negative membrane curvature (J. E. Vance & Tasseva, 2013). Phosphatidylinositol (PI) is a minor component of biological membranes, usually found on the inner (cytoplasmic) leaflet of the biological membrane. Carrying a net negative charge at physiological pH, it plays important roles in lipid signalling, cell signalling as well as membrane trafficking. Phosphatidylserine (PS) is another minor component of biological membranes except in the plasma membrane, and is involved in cell cycle signalling as well as in apoptosis. Cardiolipin (CL) is found mainly in the inner mitochondrial membrane of the mitochondria, and contribute to the optimization of numerous enzymes involved in mitochondrial energy metabolism. As phospholipids serve varying different functions, their composition in biological membranes are regulated tightly.

Being the two most abundant phospholipids in biological membranes, PC and PE have been attributed to biological membrane properties, such as membrane thickness, fluidity, saturation and curvature (Boumann et al., 2006, Dawaliby et al., 2016, J. E. Vance & Tasseva, 2013, Szule, Fuller, & Rand, 2002). Membrane proteins' activities have also been reported to be regulated by phospholipids (Yeagle, 1989). Low hepatic PC/PE ratio have been found to induce hepatic ER stress in mice (Gao et al., 2015), and hence is linked to diseases such as non-alcoholic fatty liver disease in animal models (Li et al., 2006) as well as in patients (Arendt et al., 2013). Therefore,

phospholipid homeostasis is crucial for the maintenance of various cellular processes and functions in the cell.

1.4. The biosynthesis pathway of PC is highly conserved in eukaryotes

PC is synthesised exclusively on the endoplasmic reticulum (ER) (Lagace & Ridgway, 2013). PC can be synthesised by two independent pathways. In human, PC is synthesised from PE in a 3-step *de novo* pathway by phosphatidylethanolamine N-methyltransferase (PEMT), where PEMT catalyses all three transmethylation (Figure 1.3) (J. Bremer, 1961; Z. Li & Vance, 2008). In budding yeast *Saccharomyces cerevisiae*, this reaction is first catalysed by Cho2 in the first methylation of PE to N-monomethyl phosphatidylethanolamine (MMPE) (van Meer et al., 2008; Zinser et al., 1991). Two methyl groups are further added to MMPE to synthesise PC by Opi3, the homologue of PEMT in yeast. Alternatively, PC can be synthesised from the precursor choline, obtained either by dietary consumption or by metabolism of choline-containing lipids, through the Kennedy pathway (Carman & Henry, 1989). The Kennedy pathway utilises three enzymatic steps; choline is first converted to phosphocholine, followed by the addition of cytidine-5'-triphosphate (CTP) to cytidine-diphosphocholine, and finally with diacylglycerol (DAG) to PC. Both pathways are highly conserved from yeast to humans. The *de novo* pathway accounts for a significant 30% of PC biosynthesis, while the Kennedy pathway accounting for the 70% in mouse liver (D. E. Vance, 2014). In other mammalian tissues, the activity of PEMT is drastically lower than in the liver, signifying its importance in liver. PC is then exported to other organelles through COPII-coated vesicles (Gillon, Latham, & Miller, 2012). However, the specific mechanism in which the export of PC utilised is not well characterised.

Figure 1.3. Metabolic pathways for the synthesis of phosphatidylcholine in yeast and human.

In the *de novo* pathway, PE is tri-methylated to PC by PEMT in humans, while PE is methylated to MMPE by Cho2, then methylated to DMPE and PC by Opi3. PC can be synthesised from choline via the Kennedy pathway. PE, phosphatidylethanolamine; MMPE, N-monomethyl phosphatidylethanolamine; DMPE, N,N-dimethyl phosphatidylethanolamine; PC, phosphatidylcholine; P-choline, phosphocholine; CDP-choline, cytidine diphosphate-choline.

1.5. PC/PE ratio perturbation contributes to metabolic diseases as well as other disabilities

The maintenance of PC to PE ratio is crucial for cellular viability, and studies have linked PC/PE disequilibrium to several diseased states. Firstly, PEMT knockout mice were found to develop steatohepatitis and liver failure within 3 days of choline deficient diets (Z. Li & Vance, 2008; Walkey et al., 1998; Zhu, Song, Mar, Edwards, & Zeisel, 2003). Chronic ER stress was found to be present in the liver of such mice. In another finding, chronic ER stress has been found in obese mouse liver with a PC/PE ratio higher than normal (Fu et al., 2011). A loss of function of the sarcoplasmic reticulum Ca^{2+} -ATPase pump (SERCA) was attributed to the change in phospholipid composition and suggested to be the cause of ER stress in the liver. The eventual failure of the liver and the development of NAFLD in the mice was attributed to the prolonged ER stress

faced by the liver cells under PC/PE imbalance. This indicates that both an elevated or decreased PC/PE ratio could contribute to steatohepatitis, and thus stress the importance in maintaining hepatic PC/PE homeostasis.

Decreased PC levels were also identified in muscle cells of patients to cause congenital muscular dystrophy (Mitsuhashi et al., 2011). In addition, the deficiency of Choline kinase beta which catalyses the first step in the Kennedy pathway has been identified to result in intellectual disability, autistic features, ichthyosis-like skin changes, and dilated cardiomyopathy (Haliloglu, Talim, Sel, & Topaloglu, 2015). While altered phospholipid biosynthesis was shown as a causative agent, the detailed molecular mechanisms of the diseases have not been fully elucidated.

High fat diet (HFD) has a profound impact on phospholipid composition of mice via the gut microbiome. Mice fed HFD exhibit an increased gut microbiota metabolic activity of choline (Dumas et al., 2006; Nicholson et al., 2012). This subsequently results in decreased choline bioavailability for the host. Thus, less PC is synthesised by the Kennedy pathway, resulting in symptoms that mimic NAFLD. In another study, increased dietary choline was found to promote atherosclerosis leading to cardiovascular disease (CVD) (Z. Wang et al., 2011). The abundance of the metabolites of PC, choline, betaine and trimethylamine N-oxide, are also found to be good predictors for CVD, stressing the importance of PC homeostasis and the link of PC perturbation to diseased states.

1.6. Transmembrane proteins can be affected from LP

Membrane proteins are regulated uniquely in a cell. Depending on whether the domains they possessed are cytoplasmic or luminal, distinct cellular quality control mechanisms monitor and regulate their folding states. Transmembrane proteins (TPs)

possessing domains on both sides of a membrane have quality control systems acting in unison in their regulation (Houck & Cyr, 2012). The regulation of membrane proteins is often controlled by ubiquitination which will be described in detail in the later part. Ubiquitination is a multistep enzymatic process that adds a ubiquitin moiety, or a chain of ubiquitin to the target protein. Ubiquitination acts in protein sorting, trafficking and removal of membrane proteins via endocytosis (Foot, Henshall, & Kumar, 2017). Extraction of these proteins through ubiquitin-dependent endocytosis serves to rapidly decrease their abundance which could otherwise result in diseases. Ubiquitination has also been suggested to be involved in cellular transport of membrane proteins.

Destabilising mutations in the transmembrane domains of TPs have been found to result in diseased states. Such diseases include cystic fibrosis, retinitis pigmentosa, hypercholesterolemia, diabetes insipidus, and hypogonadotropic hypogonadism (Beglova & Blacklow, 2005; Bichet, 2006; Janovick, Maya-Nunez, & Conn, 2002; Mendes, van der Spuy, Chapple, & Cheetham, 2005; O'Sullivan & Freedman, 2009; Sanders & Myers, 2004). Environmental changes such as the perturbation in PC/PE has also been known to disrupt TPs' functions. In an *in vitro* liposomes assay, varying the concentration of dioleoyl-phosphatidylethanolamine (DOPE) with dioleoyl-phosphatidylcholine (DOPC) was found to cause the aspartate transporter to flip inside-out, causing a loss in function (McIlwain, Vandenberg, & Ryan, 2015). Decrease in PE in the inner membrane of mitochondria has been reported to lead to the loss in function of the transporter outer membrane (TOM) complex (Birner, Nebauer, Schneiter, & Daum, 2003). Also in obese mouse models, it was found that PC/PE level is elevated in the liver, resulting in the loss of function of the sarcoplasmic reticulum Ca^{2+} -ATPase pump (SERCA), leading to chronic ER stress in the liver (Fu et al., 2011).

1.7. Cells exhibit ER stress under lipid perturbation and activate the unfolded protein response

The endoplasmic reticulum (ER) is critical for protein and lipid synthesis in cell. It is also responsible for glucose metabolism, lipoprotein regulation as well as calcium homeostasis. Disruption of the membrane composition of the ER has been known to disrupt such ER functions and cause ER stress. The depletion of inositol in yeast causes ER stress without the induction of misfolded proteins, indicating disturbances to membrane composition as the cause (Lajoie, Moir, Willis, & Snapp, 2012). The deletion of *mdt-15* in *Caenorhabditis elegans*, a subunit of the transcriptional regulator complex Mediator, impacted membrane lipid saturation which resulted in ER stress similarly without the induction of misfolded proteins (Hou et al., 2014). In mice model, PC/PE imbalance resulted in the destabilisation of SERCA, disrupting calcium homeostasis and resulted in ER stress (Fu et al., 2011). Hence, strong links have been found between lipid perturbation and ER stress.

The unfolded protein response (UPR) is an intracellular signalling pathway that is activated upon ER stress. It is a collective response of signal transduction pathways targeted to restore ER homeostasis from misfolded proteins. In mammalian systems, the transmembrane proteins Ire1 α , PERK and ATF6 monitor the ER lumen for the presence of unfolded proteins (Figure 1.4). Two forms of Ire1 are found in mammals where Ire1 α is expressed ubiquitously while Ire1 β is expressed solely in the intestinal and lung epithelium (Bertolotti et al., 2001; Martino et al., 2013). Ire1 α is postulated to be activated by two models. In the first model, Ire1 might be activated from the direct binding of unfolded proteins. In the second model, the molecular chaperone binding immunoglobulin protein (BiP) earlier bound to the luminal domain of Ire1 is released from Ire1 to bind unfolded proteins, hence activating Ire1. Upon activation, Ire1 α oligomerizes and phosphorylates itself, splices *XBP1* mRNA and this results in the

translation of the transcription factor XBP1 regulating downstream UPR signalling cascade. Upon prolonged ER stress, Ire1 α cleaves mRNAs to relieve protein load through its regulated Ire1-dependent decay (RIDD) activity. This cleavage of mRNAs is specific and is identified by an *XBP1*-like consensus site (Maurel, Chevet, Tavernier, & Gerlo, 2014). PERK, which is an ER transmembrane kinase, mediates transcriptional and translational control of the UPR programme (Harding, Zhang, & Ron, 1999). Upon ER stress, PERK oligomerizes and phosphorylates itself together with eIF2 α (eukaryotic initiation factor 2 α). eIF2 α phosphorylation results in temporary attenuation of the overall protein translation and up-regulation of the transcription factor ATF4 to activate downstream UPR genes. This translation inhibition resultantly decreases the influx of proteins entering the ER, reducing ER protein folding load and alleviating ER stress. Upon detection of unfolded protein accumulation, ATF6 is packaged into vesicles and transported to the Golgi apparatus (Schindler & Schekman, 2009). Cleavage of ATF6 luminal and transmembrane domain occurs subsequently by S1P and S2P proteases, liberating the N-terminal cytosolic fragment, ATF6(N), for localisation into the nucleus to activate UPR target genes (Yoshida et al., 2000).

In *S. cerevisiae*, only Ire1 is conserved as the sole UPR transducer. Upon activation, Ire1 undergoes trans-autophosphorylation and oligomerization (Figure 1.5) (Wu, Ng, & Thibault, 2014). HAC1 pre-messenger RNA is spliced by activated Ire1 through its RNase domain to initiate synthesis of Hac1 transcription. The transcription factor downstream of Ire1, Hac1, exhibits variance in its primary amino acid sequence with XBP1 but shares the common Ire1-mediated unconventional splicing activation of its mRNA and the bZIP (basic leucine zipper) motif. Hac1 is transported into the nucleus and it activates similar downstream target genes as XBP1 in mammalian systems (Patil & Walter, 2001), upregulating a broad spectrum of nearly 400 target genes which include heat shock molecular chaperones, endoplasmic reticulum-associated

degradation (ERAD), vacuolar degradation factors, lipid biosynthesis enzymes, as well as factors involved in protein translocation (Thibault, Ismail, & Ng, 2011). The upregulation of such factors aids in the folding and if necessary, the removal of misfolded proteins in the ER. Hence, ER stress can be alleviated by successful activation of the UPR.

Recent studies have indicated that the UPR can directly sense and be activated by LP. It was discovered in yeast that changes in lipid composition could also activate Ire1 in a different way from unfolded proteins (Promlek et al., 2011). IRE1 α and PERK lacking their luminal unfolded protein stress-sensing domains were also found to retain responsiveness to increased lipid saturation (Volmer, van der Ploeg, & Ron, 2013). They proposed a mechanism that LP enhanced the dimerization of IRE1 α and PERK via their transmembrane domain through increased protein-protein interactions between membrane proteins. IRE1 α and PERK is further supported in another study to be activated by increased lipid saturation in a similar fashion (Kitai et al., 2013). Recently, the molecular mechanism of how Ire1 is activated under LP in yeast is revealed, strengthening the evidence of direct sensing and activation of the UPR by LP (Halbleib et al., 2017).

Figure 1.4. The unfolded protein response in mammals.

Three different ER stress transducer, IRE1, PERK and ATF6 form the three branches of the UPR pathways in mammals. In response to ER stress, Ire1p oligomerizes and phosphorylates itself, splice XBP1 mRNA and this results in the translation of the transcription factor XBP1 regulating downstream signalling cascade. (Regulated Ire1-dependent decay) RIDD is also activated by IRE1 activation to cleave specific mRNAs to relieve protein load. PERK oligomerizes and phosphorylates itself together with eIF2α, where it attenuates protein translation. It further activates the transcription factor ATF4, which carries out downstream activation of UPR genes. Under ER stress, ATF6 is packaged into vesicles and transported to the Golgi apparatus. Cleavage of ATF6 luminal and transmembrane domain occur, where the N-terminal cytosolic fragment, ATF6(N) localise into the nucleus to activate UPR target genes. Adapted from (Wu, Ng, & Thibault, 2014).

Figure 1.5. The unfolded protein response in *Saccharomyces cerevisiae*.

Upon activation by ER stress, Ire1 undergoes oligomerization and trans-autophosphorylation, resulting in the splicing of *HAC1* through its RNase domain. Upon translation, transcription factor Hac1 transcribes UPR target genes to restore ER homeostasis. Adapted from (Wu, Ng, & Thibault, 2014).

1.8. ERAD, vacuolar degradation, lipid biosynthesis and protein translocation are upregulated by the UPR

The ERAD pathway targets misfolded proteins in the ER for degradation (Figure 1.6). Misfolded proteins can be recognised from the exposure of hydrophobic regions, unpaired cysteine residues, or immature glycans (Meusser, Hirsch, Jarosch, & Sommer, 2005). Two distinct complexes, Hrd1 and Doa10 complexes, are part of the ERAD machinery in yeast (Carvalho, Goder, & Rapoport, 2006). Proteins such as Cue1, Ubc7, and Cdc48 are common components found in both complexes (Carvalho

et al., 2006). Most proteins in the multisubunit complexes are highly conserved between yeast and mammals (Kostova, Tsai, & Weissman, 2007). Misfolded proteins are polyubiquitinated by E1, E2 and E3 enzymes. A ubiquitin-activating enzyme (E1), Uba1, adenylates a hydrolysed ATP to an ubiquitin molecule (McGrath, Jentsch, & Varshavsky, 1991). The ubiquitin molecule is then translocated to a cysteine present in a ubiquitin conjugating enzyme (E2), Ubc7 (Cohen, Wiener, Reiss, & Ravid, 2015). Lastly, ubiquitin enzymes (E3) such as Doa10 and Hrd1 catalyse the transfer of ubiquitin from E2 to misfolded protein (Ismail & Ng, 2006). Elongation occurs through a method of substrate-assisted catalysis to form a polyubiquitination chain connected in series to the protein via the E2-donor ubiquitin complex (Wickliffe, Lorenz, Wemmer, Kuriyan, & Rape, 2011). The protein is then fully extracted from the membranes by the Cdc48 AAA-ATPase complex. The polyubiquitinated protein is recognised by the 26S proteasome complex and subsequently undergoes proteolysis in the cytosol. Under ER stress, ERAD is upregulated by the UPR programme to aid in the clearance of misfolded proteins.

In addition, misfolded proteins from the ER can be delivered to the Golgi apparatus, packaged into vesicles and transported to the vacuole for degradation (Gaynor, te Heesen, Graham, Aebi, & Emr, 1994; Wang, Thibault, & Ng, 2011). This serves as an alternative mechanism in the degradation of misfolded proteins. The vacuolar degradation pathway is analogous to the lysosomal pathway in mammals (Reumann, Voitsekhovskaja, & Lillo, 2010).

Under ER stress, membrane expansion of the ER occurs to accommodate newly synthesised folding machinery, as well as to increase volume to tolerate the increase of misfolded protein concentrations and reduce aggregation (Schuck, Prinz, Thorn, Voss, & Walter, 2009). Consequently, the expanded ER alleviates stress

independently of the UPR. Phospholipid biosynthesis proteins are upregulated to expand the ER membrane (Schuck et al., 2009). The overexpression of the transcription factor for UPR genes in mammals, XBP1 (Hac1 homologue), has been found to elevate levels of ER membrane phospholipids levels (Shaffer et al., 2004; Sriburi et al., 2007; Sriburi, Jackowski, Mori, & Brewer, 2004).

Figure 1.6. Schematic representation of Hrd1 and Doa10 complexes in yeast.

The Doa10 complex recognises misfolded proteins from their cytosolic lesions (ERAD-C). The Hrd1 complex recognises misfolded proteins from lesions in their luminal domains (ERAD-L), as well as lesions within their transmembrane domains (ERAD-M). Adapted from (Thibault & Ng, 2012). *, Site of lesions in which misfolded proteins are monitored by the ERAD machinery.

Protein translocation is increased and upregulated by the UPR programme. Translocation is crucial for the transport of proteins into and across biological membranes. Protein translocation across the ER membrane can occur either co-translationally, in which insertion occurs concomitantly with protein synthesis, or post-translationally, where translocation occurs after the complete synthesis of the polypeptide. In yeast, co-translational translocation is mediated solely by the translocon, the Sec61 complex, while post-translational translocation is mediated by both Sec61 and Sec63 complexes, where Sec63 complex is a supplementary complex

(Figure 1.7) (Deshaies, Sanders, Feldheim, & Schekman, 1991). Protein translocation and the translocation channel are highly conserved from yeast to humans (Gorlich, Prehn, Hartmann, Kalies, & Rapoport, 1992). The yeast sec61 complex comprises of Sec61, the main channel for protein translocation, Sec sixty-one suppressor (Sss1), an essential subunit in the translocation complex (Esnault, Blondel, Deshaies, Scheckman, & Kepes, 1993), and Sec61 beta homologue 1 (Sbh1). Sec61 is the largest and major subunit of the heterotrimeric Sec61 complex, and forms a channel in the ER membrane. Sss1 is a small essential subunit in the Sec61 complex that interacts physically with Sec61 and stabilises the translocation channel (Esnault et al., 1994). Sbh1 is found to be non-essential for translocation (Finke et al., 1996). However, when *SBH1* gene and its paralog *SBH2* are knocked-out, a delay in co-translational translocation is reported, while a milder decrease in post-translational translocation is detected (Feng et al., 2007). Sec61 beta homologue 2 (Sbh2) shares 53% sequence similarity with Sbh1 (J. Toikkanen et al., 1996). Sbh2 forms a trimeric complex with Sec sixty-one homologue (Ssh1) and Sss1, which is reported to be involved in protein co-translational translocation into the ER as well (Finke et al., 1996). While the Sec61 complex forms the channel for both co-translational translocation as well as post-translational translocation, the Ssh1 complex is involved exclusively in co-translational translocation.

1.9. Unresolved ER stress can lead to cell death

The UPR can fail to alleviate ER stress and restore ER homeostasis in certain circumstances. Firstly, the source of ER stress can be severe or persistent, resulting in constitutive activation of the UPR. Alternately, the UPR programme can also be insufficient to alleviate ER stress when the signalling or responsive elements of UPR are dysfunctional, and misfolded proteins accumulate. In either case, it results in cell toxicity and UPR-activated apoptotic signalling cascade and consequently cell death

(Szegezdi, Logue, Gorman, & Samali, 2006). In normal physiological conditions, UPR-mediated apoptosis is initially meant to eliminate the few cells that failed to reach ER homeostasis. Non-apoptotic cells can result in inflammation, while mediated apoptosis can prevent post-apoptotic necrosis and induce anti-inflammatory response by efficient phagocytic clearance (Fadok, Bratton, & Henson, 2001). In contrast to such selective UPR mediated apoptosis, unresolved ER stress can result in the apoptosis of many cells, resulting in the development of diseased states (Kaufman, 2002). Hence, the UPR is capable of facilitating stress adaptation as well as initiating apoptosis, making this powerful stress response pathway implicated in various diseases including metabolic disease, neurodegenerative disease, inflammatory disease, and cancer (Vandewynckel et al., 2013).

Figure 1.7. Proteins forming the Sec61 and Sec61/63 complexes in co-translational and post-translational translocation machineries.

Proteins forming the Sec61 complex involved in co-translational translocation are Sec61, Sbh1 and Sss1. Proteins forming the Sec61/63 complex involved in post-translational translocation are proteins in the Sec61 complex, as well as Sec62, Sec63, Sec66, Sec72 and Kar2 in the Sec63 complex.

1.10. Previous works revealed a broad remodelling of protein homeostasis networks in response to LP

Using *Saccharomyces cerevisiae*, our lab has genetically modified yeast to mimic the PC/PE imbalance, by deleting the genes *CHO2* or *OPI3* (Thibault et al., 2012). *CHO2* or *OPI3* knockouts cells block PC synthesis from PE through the *de novo* pathway. Using minimal synthetic media without choline supplementation, the synthesis of PC could be effectively blocked in the cell. Despite having important roles in synthesizing PC from PE, *CHO2* or *OPI3* is not essential for cell survival. Ablation of *CHO2* or *OPI3* genes was found to subsequently reduce PC by 2.8 folds and 173 folds respectively (Figure 1.8), with PC being virtually absent in *OPI3* knockout cells. The reduction but not elimination of PC in *CHO2* knockout cells indicate that Opi3 partially compensate in the first methylation of PE to MMPE of Cho2 (Kodaki & Yamashita, 1987). The human homologue of *OPI3*, PEMT catalyses all three transmethyations (Kanipes & Henry, 1997). PE levels were found to decrease in *opi3Δ* mutant with *CHO2* being active, resulting in an accumulation of MMPE, the first methylated product of PE.

The disruption of PC/PE homeostasis affects multiple cellular pathways and organelles (Thibault et al., 2012). Therefore, a global approach was taken by our lab to understand the cellular response to lipid disequilibrium. Genomic changes under lipid disequilibrium were obtained using DNA microarray, while proteomic and lipidomic profiles of cells were obtained using mass spectrometry. A broad remodelling of the protein homeostasis networks is reported in response to lipid disequilibrium (Thibault et al., 2012) (Figure 1.9). Upon activation of UPR by LP, major systems were downregulated. This includes ribosomal proteins, assembly and nuclear transport factors, translation initiation factors etc. involved in general protein synthesis and regulation. Hence general protein attenuation occurs. However, an upregulation was observed in proteins involved in ERAD, vacuolar degradation pathway, secretory

pathway, heat shock response (HSR), the unfolded protein response (UPR), as well as proteins involved in co-translational and post-translational translocation. Surprisingly, minimal changes in the lipidome were detected in lipid perturbed cells, establishing remodelling of proteostasis as a major adaptation to LP. Hence, looking at changes in the protein homeostasis networks would allow us to understand how cells are affected by LP and their response.

Figure 1.8. *cho2Δ* and *opi3Δ* cells have lower PC levels than in WT cells.

Bar graph comparing total PE, MMPE and PC in wild type (WT), *cho2Δ* and *opi3Δ* cells. PC levels are reduced in *cho2Δ* cells and virtually absent in *opi3Δ* cells as compared to WT. PE level is increased in *cho2Δ* but decreased in *opi3Δ* cells compared to WT, while accumulation of MMPE is detected in *opi3Δ* cells. PE, phosphatidylethanolamine; MMPE, N-monomethyl phosphatidylethanolamine; PC, phosphatidylcholine. Adapted from (Thibault et al., 2012).

Figure 1.9. Changes in protein expression of cells in response to lipid disequilibrium.

Changes in the protein expression of *cho2Δ* and *opi3Δ* cells compared to WT are illustrated by colour coding. Green signifies a decrease in protein expression while red signifies an increase. Adapted from (Thibault et al., 2012).

1.11. Certain proteins were found to be transcriptionally upregulated but exhibited lower protein abundance

We explored deeper into the profile of cells under lipid disequilibrium. Overall, the data from the DNA microarray analysis and the proteomic analysis in *opi3Δ* cells correlate well; an increase in the transcription of a protein matches an increase in the protein steady state level and *vice versa* (Figure 1.10). However, certain outliers were identified where the proteins were found to be in low abundance while their corresponding mRNA was found to be transcriptionally upregulated (Figure 1.10, yellow box). This suggests that the proteins were either downregulated post-transcriptionally, or they were degraded more rapidly and have shorter half-lives. As

some proteins were found to be involved in alleviating ER stress, we began this study to understand how this phenomenon and its effect on the cells.

Figure 1.10. Correlation of relative protein and mRNA abundance under lipid disequilibrium.

Pearson correlation of relative protein and mRNA abundance in *opi3Δ* compared to WT. Proteins that are transcriptionally upregulated but are in lower abundance are found in the yellow quadrant. R, Pearson correlation; P, *P* value; n, sample number. Adapted from (Thibault et al., 2012).

1.12. Thesis objectives

Despite recent advances made in the understanding of lipid homeostasis and the importance of their tight regulation, the biological significance of membrane homeostasis remains largely unclear. The complex organization of cellular membranes suggests equally complex homeostatic regulatory mechanisms, but how such mechanisms fail and result in the development of metabolic diseases remain largely unexplored. LP provides a link between how obesity could result in metabolic diseases

such as NAFLD and T2D. This study aims to develop a model of how LP could result in unresolved ER stress that could develop into such diseased states.

From earlier work, we have demonstrated that cells respond to lipid perturbation through major changes in the protein homeostatic networks, with minimal changes to the lipidome (Thibault et al., 2012). This established the upregulation in protein quality control pathways such as the UPR is essential for cells to adapt to lipid stress. A concerted investigation into both the genomics and proteomics of the cell surprisingly revealed a subset of ER transmembrane proteins (TPs) which are part of the UPR programme to be prematurely degraded (Chapter 3). As such phenomenon can have adverse effects on the cell, we moved on to understand what causes their premature degradation. Our study revealed that ER membrane have undergone fatty acid remodelling and membrane stiffening which destabilised the TPs. To reveal how TPs became prematurely degraded under LP, we used Sbh1 as a model to investigate this mechanism (Chapter 4). Interestingly, Sbh1 was also found to play a role in protein processing. Together, my studies serve to further advance our understanding of how cells adapt under LP, and revealed that LP could compromise UPR-mediated ER homeostasis. This can explain the onset of NAFLD, where cells fail to fully restore ER homeostasis under LP, resulting in chronic ER stress. In addition to this application, disrupting phospholipid homeostasis may be exploited to target pathogens like *Plasmodium falciparum* that upregulate the UPR for survival. Two novel lipid genes, *PfFMP* and *PfPSD* were characterised in *Plasmodium falciparum* by genetic complementation in yeast (Chapter 5).

Chapter 2. Material and methods

2.1. Statistics

Error bars indicate standard error of the mean (SEM), calculated from at least three biological replicates, unless otherwise indicated. *P* values were calculated using two-tailed Student's *t* test, unless otherwise indicated, and reported as *P*=value in figures.

2.2. Strains and antibodies

Saccharomyces cerevisiae strains used in this study are listed in Table 2.1. Strains were generated using standard cloning protocols. Anti-CPY polyclonal rabbit antibody, anti-Gas1-N_T polyclonal rabbit antibody, anti-Kar2 polyclonal rabbit antibody and anti-Sec61 polyclonal rabbit antibody were gifts from Davis Ng (Temasek Life Sciences Laboratories, Singapore). Anti-HA mouse monoclonal antibody HA.11 (Covance, Princeton, NJ), anti-Pgk1 mouse monoclonal antibody (Invitrogen), anti-tubulin mouse monoclonal antibody 12G10 (DHSB, Iowa City, Iowa) and anti-LexA polyclonal rabbit antibody (Abcam, Cambridge, United Kingdom) were commercially purchased. Secondary antibodies goat anti-mouse IgG-DyLight 488 (Thermo Fisher, Waltham, MA), anti-rabbit IgG-DyLight 550 (Thermo Fisher, Waltham, MA), goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, Dallas, TX), goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Dallas, TX), goat anti-mouse IgG-IRDye 800 (LI-COR Biosciences) and goat anti-rabbit IgG-IRDye 680 (LI-COR Biosciences, Lincoln, NE) were commercially purchased.

Table 2.1. Yeast strains used in the study.

Strains	Genotype	Source
W303a	<i>MATa, leu2-3,112, his3-11, trp1-1, ura3-1, can1-100, ade2-1</i>	(Cox, Shamu, & Walter, 1993)
GTY68	<i>MATa, opi3::KANMX</i> , W303 background	(Thibault et al., 2012)
YGT0294	<i>MATa</i> , pGT0196, W303 background	This study
YGT0296	<i>MATa, opi3::KANMX</i> , pGT0196, W303 background	This study
YGT0300	<i>MATa</i> , pGT0198, W303 background	This study
YGT0302	<i>MATa, opi3::KANMX</i> , pGT0198, W303 background	This study
YGT0315	<i>MATa</i> , pGT0181, W303 background	This study
YGT0317	<i>MATa, opi3::KANMX</i> , pGT0181, W303 background	This study
YGT0318	<i>MATa</i> , pGT0182, W303 background	This study
YGT0320	<i>MATa, opi3::KANMX</i> , pGT0182, W303 background	This study
YGT0321	<i>MATa</i> , pGT0179, W303 background	This study
YGT0323	<i>MATa, opi3::KANMX</i> , pGT0179, W303 background	This study
YGT0327	<i>MATa</i> , pGT0185, W303 background	This study
YGT0329	<i>MATa, opi3::KANMX</i> , pGT0185, W303 background	This study
YGT0330	<i>MATa</i> , pGT0315, W303 background	This study
YGT0332	<i>MATa, opi3::KANMX</i> , pGT0315, W303 background	This study
YGT0374	<i>MATa</i> , pGT0183, W303 background	This study
YGT0375	<i>MATa, opi3::KANMX</i> , pGT0183, W303 background	This study
YGT0412	<i>MATa</i> , pRS315, pRS316, W303 background	This study
YGT0413	<i>MATa, ire1::trp1</i> , pRS315, pRS316, W303 background	This study
YGT0414	<i>MATa, cho2::KANMX</i> pRS315, pGT55, W303 background	This study
YGT0415	<i>MATa, cho2::KANMX, ire1::trp1</i> , pRS315, pGT55, W303 background	This study
YGT0421	<i>MATa, opi3::KANMX</i> , pGT0263, W303 background	This study
YGT0422	<i>MATa</i> , pGT0263, W303 background	This study
YGT0432	<i>MATa</i> , pJC835, W303 background	This study
YGT0497	<i>MATa</i> , pGT0281, pRS316, W303 background	This study
YGT0498	<i>MATa, ire1::trp1</i> , pGT0281, pRS316, W303 background	This study
YGT0499	<i>MATa, cho2::KANMX</i> pGT0281, pGT55, W303 background	This study
YGT0500	<i>MATa, cho2::KANMX, ire1::trp1</i> , pGT0281, pGT55, W303 background	This study
YGT0540	<i>MATa</i> , pGT0288, NMY51 background (<i>his3Δ200, trp-901, leu2-3,112, ade2, LYS::(lexAop)4-HIS3, ura3::(lexAop)8-LACZ, (lexAop)8-ADE2, GAL4</i>)	This study
YGT0541	<i>MATa, opi3::KANMX</i> , pGT0183, NMY51 background	This study
YGT0574	<i>MATa, doa10::KANMX, opi3::KANMX</i> , pGT0183, W303 background	This study
YGT0575	<i>MATa, hrd1::KANMX, opi3::KANMX</i> , pGT0183, W303 background	This study
YGT0576	<i>MATa, usa11::KANMX, opi3::KANMX</i> , pGT0183, W303 background	This study
YGT0577	<i>MATa</i> , pRS315, W303 background	This study
YGT0580	<i>MATa</i> , pGT0199, W303 background	This study
YGT0582	<i>MATa, opi3::KANMX</i> , pGT0199, W303 background	This study

Strains	Genotype	Source
YGT0586	<i>MATa, psd1::KANMX</i> , pRS315, W303 background	This study
YGT0587	<i>MATa, fmp30::KANMX</i> , pRS315, W303 background	This study
YGT0588	<i>MATa, fmp30::KANMX</i> , pGT0323, W303 background	This study
YGT0589	<i>MATa, fmp30::KANMX, psd1::KANMX</i> , pRS315, W303 background	This study
YGT0590	<i>MATa, fmp30::KANMX, psd1::KANMX</i> , pGT0323, W303 background	This study
YGT0598	<i>MATa</i> , pRS315, pRS316, pDN314, W303 background	This study
YGT0599	<i>MATa, ire1::trp1</i> , pRS315, pRS316, pDN314, W303 background	This study
YGT0600	<i>MATa, cho2::KANMX</i> pRS315, pGT55, pDN314, W303 background	This study
YGT0601	<i>MATa, cho2::KANMX, ire1::trp1</i> , pRS315, pGT55, pDN314, W303 background	This study
YGT0604	<i>MATa</i> , pGT0281, pRS316, pDN314, W303 background	This study
YGT0605	<i>MATa, ire1::trp1</i> , pGT0281, pRS316, pDN314, W303 background	This study
YGT0606	<i>MATa, cho2::KANMX</i> , pGT0281, pGT55, pDN314, W303 background	This study
YGT0607	<i>MATa, cho2::KANMX, ire1::trp1</i> , pGT0281, pGT55, pDN314, W303 background	This study
YGT0649	<i>MATa, fmp30::KANMX</i> , pRS315, pRS316, W303 background	This study
YGT0650	<i>MATa, fmp30::KANMX</i> , pGT0323, pRS316, W303 background	This study
YGT0651	<i>MATa, psd1::KANMX</i> , pRS315, pRS316, W303 background	This study
YGT0652	<i>MATa, psd1::KANMX</i> , pRS315, pGT0324, W303 background	This study
YGT0653	<i>MATa, fmp30::KANMX, psd1::KANMX</i> , pRS315, pRS316, W303 background	This study
YGT0654	<i>MATa, fmp30::KANMX, psd1::KANMX</i> , pGT0323, pRS316, W303 background	This study
YGT0655	<i>MATa, fmp30::KANMX, psd1::KANMX</i> , pRS315, pGT0324, W303 background	This study
YGT0656	<i>MATa, fmp30::KANMX, psd1::KANMX</i> , pGT0323, pGT0324, W303 background	This study
YGT0671	<i>MATa</i> , pGT0352, W303 background	This study
YGT0672	<i>MATa, opi3::KANMX</i> , pGT0352, W303 background	This study
YGT0673	<i>MATa</i> , pGT0183, pRS313, W303 background	This study
YGT0674	<i>MATa, opi3::KANMX</i> , pGT0183, pRS313, W303 background	This study
YGT0675	<i>MATa</i> , pGT0183, pGT0349, W303 background	This study
YGT0676	<i>MATa, opi3::KANMX</i> , pGT0183, pGT0349, W303 background	This study
YGT0690	<i>MATa</i> , pGT0180 , W303 background	This study
YGT0691	<i>MATa, opi3::KANMX</i> , pGT0180, W303 background	This study
YGT0721	<i>MATa</i> , pGT0350, pGT0183, W303 background	This study
YGT0722	<i>MATa, opi3::KANMX</i> , pGT0350, pGT0183, W303 background	This study
YGT0725	<i>MATa</i> , pGT0350, pRS315, W303 background	This study
YGT0726	<i>MATa, opi3::KANMX</i> , pGT0350, pRS315, W303 background	This study
YGT0769	<i>MATa</i> , pGT0366, W303 background	This study

Strains	Genotype	Source
YGT0770	<i>MATa, opi3::KANMX</i> , pGT0366, W303 background	This study
YGT0771	<i>MATa</i> , pGT0368, W303 background	This study
YGT0772	<i>MATa, opi3::KANMX</i> , pGT0368, W303 background	This study
YGT0773	<i>MATa</i> , pGT0365, W303 background	This study
YGT0774	<i>MATa, opi3::KANMX</i> , pGT0365, W303 background	This study
YGT0874	<i>MATa</i> , pPS1622, pRS313, W303 background	This study
YGT0875	<i>MATa, opi3::KANMX</i> , pPS1622, pRS313, W303 background	This study
YGT0876	<i>MATa</i> , pGT0349, pPS1622, W303 background	This study
YGT0877	<i>MATa, opi3::KANMX</i> , pGT0349, pPS1622, W303 background	This study
YGT885	<i>MATa, fmp30::KANMX</i> , pGT0408, pRS316, W303 background	This study
YGT889	<i>MATa, fmp30::KANMX, psd1::KANMX</i> , pGT0408, pRS316, W303 background	This study
YGT0891	<i>MATa, fmp30::KANMX, psd1::KANMX</i> , pGT0408, pGT0324, W303 background	This study
YGT947	<i>MATa, fmp30::KANMX, psd1::KANMX</i> , pGT0412, pRS316, W303 background	This study
YGT948	<i>MATa, fmp30::KANMX, psd1::KANMX</i> , pGT0412, pGT0324, W303 background	This study

2.3. Plasmids used in this study

Plasmids and primers used in this study are listed in Table 2.2 and 2.3, respectively. Plasmids were constructed using standard cloning protocols. All coding sequences of constructs used in this study were sequenced in their entirety. The plasmid pGT0179, pGT0181, pGT0183, and pGT0185, were generated by amplifying the promoter, open reading frame, and terminator of *NSG2*, *CUE1*, *SBH1*, and *EMC4* with primer pairs BN033-034, BN029-030, BN035-036, and BN031-032, respectively, from the template WT genomic DNA (gDNA). PCR products of *NSG2*, *SBH1*, and *EMC4* were digested with the restriction enzymes *NotI* and *NcoI* before being ligated into the corresponding restriction sites in pRS315. *CUE1* PCR product was digested with the restriction enzymes *NotI* and *PstI* before being ligated into the corresponding restriction sites in pRS315. The plasmid pGT0196, pGT0198, pGT0199, and pGT0263, were generated similarly by amplifying the open reading frame, and terminator of *CUE1*, *NSG2*, *SBH1*, and *EMC4* respectively, from the template WT genomic DNA (gDNA). PCR products of *NSG2*, *SBH1*, and *EMC4* were digested with the restriction enzymes *SalI* and *BamHI*

before being ligated into the corresponding restriction sites in pRS315 containing the *PGK1* promoter. *CUE1* PCR product was digested with the restriction enzymes NotI and PstI before being ligated into the corresponding restriction sites in pRS315 containing the *PGK1* promoter. The plasmid pGT281 was generated by amplifying the open reading frame, and terminator of *SBH1* from the template WT genomic DNA (gDNA). PCR product of *SBH1* was digested with the restriction enzymes SalI and BamHI before being ligated into the corresponding restriction sites in pRS315 containing the *GPD* promoter. The plasmid pGT0288 was generated by amplifying the open reading frame of *Sbh1* with primer BN027 and BN028 from WT gDNA and digested with the restriction enzyme *Sfi*I before being ligated into the corresponding restriction sites in pBT3N. The plasmid pGT0349 was generated by Gibson assembly to join promoter of *PRC1* and open reading frame of *OLE1* generated with primers BN001 and BN002 from WT gDNA with a 3X FLAG tag amplified with primers BN015 and BN016 from pGT0284 into pRS313. The plasmid pGT0352 was generated by performing a site directed mutagenesis with primer BN037 and BN038 on pGT0183 as previously described (Nelson, Lawson, Klingenberg, & Douglas, 1993). The plasmid pGT0350 was generated by Gibson assembly to join the promoter and open reading frame of *SSS1* with primers BN013 and BN014 from WT gDNA with a 3X FLAG tag amplified with primers BN015 and BN016 from pGT0284 into pRS313. pGT0323 and pGT0324 was synthesised by GeneScript in *Escherichia coli* and codon optimized for *Saccharomyces cerevisiae*. The plasmid pGT0412 was generated by Gibson assembly to join the MLS and open reading frame of *Fmp30* with primers BN041 and BN042 from WT gDNA into pGT0323 amplified with primers BN039 and BN040. The plasmid pGT0408 was generated by performing a site directed mutagenesis with primer BN043 and BN044 on pGT0412.

Table 2.2. List of plasmids used in this study.

Plasmid	Encoded protein	Promoter	Vector	Source
pJC31	β -galactosidase	<i>UPRC-CYC1</i>	pRS315	(Cox & Walter, 1996)
pPS1622	Sec63-sGFP	<i>SEC63</i>	pRS316	(Prinz et al., 2000)
pJC835	Hac1	<i>HAC1</i>	pRS313	(E. D. Spear & Ng, 2003)
pGT0284	IRE1-3X FLAG	<i>IRE1</i>	pRS426	(Kimata, Oikawa, Shimizu, Ishiwata-Kimata, & Kohno, 2004)
pPM28	eroGFP	<i>GAP</i>	pRS316	(Merksamer, Trusina, & Papa, 2008)
pDN314	D _{HC} - α -F	<i>MF(ALPHA)1</i>	pRS313	(Ng, Brown, & Walter, 1996)
pGT55	Cho2(E25K, F70S, D190G, V546A, H713Y, S841F)	<i>CHO2</i>	pRS316	(Thibault et al., 2012)
pGT0179	Nsg2-HA	<i>NSG2</i>	pRS315	This study
pGT0181	Cue1-HA	<i>CUE1</i>	pRS315	This study
pGT0183	Sbh1-HA	<i>SBH1</i>	pRS315	This study
pGT0185	Emc4-HA	<i>EMC4</i>	pRS315	This study
pGT0196	Cue1-HA	<i>PGK1</i>	pRS315	This study
pGT0198	Nsg2-HA	<i>PGK1</i>	pRS315	This study
pGT0199	Sbh1-HA	<i>PGK1</i>	pRS315	This study
pGT0263	Emc4-HA	<i>PGK1</i>	pRS315	This study
pGT0281	Sbh1-HA	<i>GPD</i>	pRS315	This study
pGT0288	Cub-LexA-VP16-Sbh1	<i>CYC1</i>	pBT3-N	This study
pGT0323	$\Delta fmp30_M$ -PfFMP	<i>FMP30</i>	pRS315	This study
pGT0324	Psd1 _M -PfPSD-3X HA	<i>PSD1</i>	pRS316	This study
pGT0349	Ole1-3XFlag	<i>PRC1</i>	pRS313	This study
pGT0352	Sbh1(K41A)-HA	<i>SBH1</i>	pRS315	This study
pGT0350	Sss1-3XFlag	<i>SSS1</i>	pRS313	This study
pGT0408	Fmp30 _M - PfFMP-3X HA	<i>FMP30</i>	pRS315	This study
pGT0412	Fmp30 _M - PfFMP	<i>FMP30</i>	pRS315	This study

Table 2.3. Oligonucleotide primers used in this study.

Primer	Sequence (5' to 3')
BN001	AGTATACATACGGATCCATGCCAACTTCTGGAACCTAC
BN002	GTAGTCCGCATGCCCTTAAAAGAACTTACCAGTTTCG
BN013	AGTATACATACGGATCCATGGCTAGAGCTAGTGAAAAAG
BN014	GTAGTCCGCATGCCCTTAAACAATAACGTATCTGATTG
BN015	GGGCATGCGGACTACAAA
BN016	GGATCCGTATGTATACTTTAAGTTGAG
BN017	AGCTTTCCACCTGGGTTTGAGC
BN018	CGCCACCAGCAGCGTTATTATC
BN019	ACTGCCCTCATGCTACTCTCTG
BN020	ACCCAACACTGGTTTGAATGCAC
BN021	TCTGATGAGGCTACGGGACTAAG
BN022	AACCGACCGCTAGAAACAACAC
BN023	GGAGAACCGGATCCAAAGAAGAGC
BN024	AAGCTGCGGTTTAGTCAGAGAGC
BN025	TGGATTCTGAGGTTGCTGCTTTGG
BN026	ACCTTGGTGTCTTGGTCTACCG
BN027	ATGAGGGCCATTACGGCCATGTCAAGCCCAACTCCTCC
BN028	CTCATGGCCGAGGCGGCCCTTAAAATAACTTACCGGCAACTTTAGAATAACATG
BN029	CTCATGCTGCAGATGGAGGATTCGAGATTGCTTATCACTTTG
BN030	CTCATGCCATGGGAGTCAGCAAACCTTTGCAAATCTTTATCAC
BN031	AACGTCGCGGCCGCGCAGCAAATGATTCCTCGACTGAATATAAAGG
BN032	CCATGGCGCGCTAATCGGAAAACCATTTGTAATCCATTATTATAATGAGCA
BN033	CTCATGCTGCAGATGGCCAATAGAGGAGAACCGG
BN034	CTCATGCCATGGGATGAGAATATAGATATCTTCCTAGTTTTCCAAACA
BN035	CTCATGCTGCAGATGTCAAGCCCAACTCCTCC
BN036	CTCATGCCATGGGAAATAACTTACCGGCAACTTTAGAAATAACATG
BN037	AATTCGATTTTGGCGATTTATTCTGAT
BN038	ATTGCTGTTCTGTGTTTTCTTTGGAGC
BN039	CTGCAGATGAAGAAGGAC
BN040	AGATCTTTGCAACACAAGC
BN041	CTTGTGTTGCAAAGATCTATGAATTTTGTTACCTGCC
BN042	GTCCTTCTTCATCTGCAGATGTCTTTTGAAAGTTGACAATG
BN043	GCCATGGCCTACCCATAT
BN044	GGATACTATTTGGAATTCATCAAGTC

2.4. Cycloheximide chase assay

Cycloheximide chase assay was carried out as previously described (Prasad, Kawaguchi, & Ng, 2010). Typically, 6 OD₆₀₀ units of early log phase cells were grown in synthetic media. Protein synthesis was inhibited by adding 200 µg/ml cycloheximide. Samples were taken at designated time points. Cell lysates from these samples were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. Immunodetection was performed with appropriate primary antibodies and horseradish

peroxidase-conjugated secondary antibodies or IRDye-conjugated secondary antibodies. Proteins were visualized using the ECL system (C-DiGit Chemiluminescent Western Blot Scanner) or the NIR fluorescence system (Odyssey CLx Imaging System). Values for each time point were normalised using anti-Pgk1 or anti-Tub1 as loading controls. Quantification was performed using an Odyssey infrared imaging programme (LI-COR Biosciences, Lincoln, NE).

2.5. Indirect immunofluorescence

Indirect immunofluorescence was carried out as previously described (Spear & Ng, 2003). Typically, cells were grown to early log phase at 30°C in selective synthetic complete media, fixed in 10% formaldehyde and permeabilized. After blocking with 3% BSA, staining was performed using anti-HA (1:200) and anti-Kar2p primary antibody (1:1,000) followed by Alexa Fluor 488 goat anti-rabbit secondary antibody (1:1,000). Samples were visualized using a Zeiss LSM 710 microscope with a 100x 1.4 NA oil Plan-Apochromat objective (Carl Zeiss MicroImaging).

2.6. Alkaline carbonate extraction

Alkaline Carbonate Extraction was carried out as previously described (S. Wang, Thibault, & Ng, 2011). Five OD₆₀₀ of early log phase cells were resuspended in 1.2 ml of 10 mM sodium phosphate pH 7.0, 1mM Phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (PIC). Equal volume of 0.2 M sodium carbonate (pH 11.0) was added to cell lysates incubated 30 min at 4°C and spun down at 100,000 x *g* for 30 min, 4°C. The pellet (membrane fraction) was solubilized in 3% SDS, 100 mM Tris, pH 7.4, 3 mM DTT and incubated at 100°C for 10 min. Total lysate and supernatant (collected from centrifuged lysate) were precipitated with 10% trichloroacetic acid (TCA) and spun down 30 min at 18,400 x *g*, 4°C. Proteins were resuspended in TCA resuspension buffer (100 mM Tris-HCL pH 11.0, 3% SDS).

2.7. Proteinase K digestion assay

Fifty OD₆₀₀ units of early log phase cells were pelleted and resuspended in 1 ml Tris Buffer (50 mM Tris pH 7.4, 50 mM NaCl, 10% glycerol, 1mM PMSF and PIC). Clarified cell lysate was spun down at 100,000 x *g* for 1 h at 4 °C. The pellet was resuspended and washed with 0.5 ml Tris Buffer without PMSF and PIC. Around ~ 5 OD₆₀₀ equivalent of microsomes were incubated with 1 mg/ml Proteinase K (Promega, Fitchburg, WI) and 1% Nonidet P40 substitute (Sigma-Aldrich, St. Louis, MO) when indicated and incubated at 37°C for 30 min. To quench the reaction, 5 mM PMSF was added followed by TCA precipitation. Samples were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane. Immunodetection was performed with appropriate primary antibodies and IRDye-conjugated secondary antibodies. Immunoreactive species were visualized using the NIR fluorescence system (Odyssey CLx Imaging System).

2.8. Lipid extraction and fatty acid analysis

For whole cells, 10 OD₆₀₀ of early log phase cells were pelleted, washed and resuspended with ice-cold water and lyophilised using Virtis Freeze Dryer under vacuum. For lipid extraction for microsomes, 50 OD₆₀₀ of early log phase cells were pelleted, washed with phosphate-buffered saline (PBS) and resuspended in 1 ml of Tris Buffer (50 mM Tris-HCL, 150 mM NaCl, 5 mM EDTA pH 8.0, 167 µM PMSF and PIC). Clarified lysate was spun down at 100,000 x *g* for 1 h at 4°C. The pellet was resuspended in 100 µl ddH₂O and sonicated for 30 min. Lipid content was normalised to protein content using bicinchoninic acid (BCA) protein assay (Sigma-Aldrich, St. Louis, MO). Normalised microsome contents were resuspended with ice-cold ddH₂O and lyophilized using Virtis Freeze Dryer under vacuum. Lyophilised samples were subjected to 300 µl 1.25 M HCl-MeOH (Sigma-Aldrich, St. Louis, MO) and incubated at

80°C for 1 h to hydrolyse and esterify FAs into FA methyl ester (FAME). FAME were extracted three times with 1 ml of hexane and separated on a gas chromatography with flame ionization detector (GC-FID; GC-2014; Shimadzu, Kyoto, Japan) equipped with an Ulbon HR-SS-10 capillary column (nitrile silicone, 25 m x 0.25 mm; Shinwa Chemical Industries, Kyoto, Japan). The temperature was held 3 min at 160°C and increase to 180°C with 1.5°C/min increments and to 220°C with 4°C/min increments.

For thin layer chromatography, dried lipid extracts were reconstituted in 50 µl of chloroform:methanol (2:1), and spotted on well-dried silica thin layer chromatography (TLC) plates (Merck Millipore) using Linomat 5 (Camag). To separate cardiolipins from other lipid species, chromatograms were developed using chloroform:hexane:methanol:acetic acid (50:30:10:5) as the solvent system. Plates were dried before application of primuline solution (0.05 mg/ml in acetone:water, 80:20).

2.9. Fluorescent recovery after photobleaching

Fluorescent recovery after photobleaching (FRAP) was carried out as previously described (Shibata et al., 2008). Typically, early log phase cells expressing Sec63-GFP were fixed on coverslips in Attofluor cell chamber (Thermo Fisher, Waltham, MA) with Concanavalin A before rinsing thrice with ddH₂O. Cells were imaged for 5 s followed by photobleaching a region of interest of 82 x 82 pixels at 100% intensity 488 nm laser under 5x magnification. Subsequently, images were taken at 1.57 sec intervals for a total of 160 sec. Images were acquired using a Zeiss LSM 710 microscope with a 100x 1.4 NA oil Plan-Apochromat objective (Carl Zeiss MicroImaging) with argon laser line 488 nm of optical slices 4.2 µm. ZEN black edition was used for image acquisition and analysis. Magnification, laser power, and detector gains were identical across samples. For data analysis, the fluorescence intensity of

three regions of interest was measured for the entire course of the experiment: the region of interest (ROI), a region outside of the cell to measure the overall background fluorescence (BG), and a non-photobleached region within the cell was monitored to measure the overall photobleaching and fluorescence variation (REF). Normalised fluorescence intensity $[F(t)_{norm}]$ was calculated for each time point using Eq. 1 (Day, Kraft, Kang, & Kenworthy, 2012). $F(i)$ denotes the initial fluorescence intensities.

$$F(t)_{norm} = \frac{F(t)_{ROI} - F_{BG}}{F(t)_{REF} - F_{BG}} \times \frac{F(i)_{REF} - F_{BG}}{F(i)_{ROI} - F_{BG}} \quad (1)$$

Fluorescent recovery was analysed by calculating half fluorescent intensity ($t_{1/2}$) using Eq. 2 (Feder, Brust-Mascher, Slattery, Baird, & Webb, 1996). F_0 denotes the normalised initial fluorescence intensity, F_{∞} the normalised maximum fluorescence intensity and $F(t)$ the normalised fluorescent intensity at each time point.

$$F(t) = \frac{F_0 + F_{\infty} \frac{t}{t_{1/2}}}{1 + \frac{t}{t_{1/2}}} \quad (2)$$

The $t_{1/2}$ values were plotted using GraphPad Prism 5.0.

2.10. Membrane yeast two-hybrid system assay

Membrane yeast two-hybrid (MYTH) assay was carried out as previously described (Snider, Kittanakom, Damjanovic, et al., 2010). Yeast two-hybrid screen uses the split ubiquitin two hybrid (N-terminus, Nub and C-terminus, Cub). Briefly, MYTH bait was generated by integrating Cub-LexA-VP16 tag at the N-terminus of Sbh1 under the control of the promoter *CYC1* and transformed in NMY51 strain. Sbh1 tagged protein localisation was verified by indirect immunofluorescence using anti-LexA antibodies against the tag as describe above. Seven μ g of NubG-X cDNA prey library (Dualsystems) was transformed in 35 OD₆₀₀ unit of *SBH1* reporter strain. Interactors

were isolated on selective complete (SC) media lacking tryptophan, leucine, adenine and histidine complemented with 80 µg/ml X-Gal and 5 mM 3-Amino-1,2,4-triazole (3-AT) and grown for two days at 30°C. The histidine inhibitor 3-AT was used to reduce false positive colonies. Only colonies which display robust growth on selective media and a blue colour were selected for further analysis. The prey cDNA plasmids were isolated and sequenced. The list of interactors was verified via the Bait Dependency Test, where all of the identified interactors are retransformed back into the original bait strain, together with a negative control using the single-pass transmembrane domain of human T-cell surface glycoprotein CD4 tagged to Cub-LexA-VP16 MYTH (Snider, Kittanakom, Curak, et al., 2010). Yeast carrying the artificial bait and prey which cause activation of the reporter system, were removed from the list of interactors. Yeast that harbour the prey and the bait-of-interest and did not grow were likewise removed from the list of interactors.

2.11. Co-immunoprecipitation

Co-immunoprecipitation was carried out as previously described (Xu & Reed, 1998). Briefly, thirty OD₆₀₀ unit of early log phase cells were incubated with 100 µg/ml dimethyl 3,3'-dithiopropionimidate dihydrochloride (DTBP) for 30 min at RT and stopped with ice-cold 50 mM TBS with 0.1% Tween 20. Cells were resuspended in 700 µl Tris buffer (50 mM Tris-HCl pH 7.9, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, mM PMSF and PIC). Clarified cell lysate was incubated with Protein G beads and anti- HA antibodies overnight at 4°C. Beads were washed thrice with Tris buffer and twice with TBS. Proteins were separated using SDS-PAGE and visualized by immunoblot as described above.

2.12. Cell Labelling and immunoprecipitation

Cell labelling and immunoprecipitation was carried out as previously described (Thibault et al., 2011). In brief, three OD₆₀₀ units of early log phase cells were labelled with 80 µCi of L-[³⁵S]-methionine/cysteine mix (Perkin Elmer) for 5 minutes. A chase was carried out with the addition of cold methionine/cysteine (2 mM final concentration) and samples were taken at designated time points. Cell lysates from these samples were then subjected to immunoprecipitation with the appropriate antiserum, resolved by SDS-PAGE and visualized using a Typhoon TRIO phosphorimager (Amersham).

2.13. β-galactosidase reporter assay

The β-galactosidase reporter assay was carried out as previously described (Thibault et al., 2011). Typically, 4 OD₆₀₀ units of early log phase cells were collected and resuspended in 75 µl LacZ buffer (125 mM sodium phosphate, pH 7, 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol). As positive control to induce the UPR, tunicamycin was added at a concentration of 2.5 µg/ml to growing WT cells 1h prior to harvest. An aliquot of 25 µl cell resuspension was transferred into 975 µl ddH₂O and the absorbance was measured at 600 nm. To the remaining resuspension, 50 µl chloroform and 20 µl 0.1% SDS were added and vortexed vigorously for 20 sec. The reaction was started with the addition of 1.4 mg/ml ONPG (2-nitrophenyl -D-galactopyranoside; Sigma) in LacZ buffer. Then, the reaction was stopped with 500 µl of 1 M Na₂CO₃ when sufficient yellow colour had developed without exceeding a ten-minute reaction. The absorbance was measured at 420 and 550 nm. The β-galactosidase activity was calculated using Eq. (3).

$$\text{Miller units} = 1000 \times (\text{OD}_{420} - 1.75 \times \text{OD}_{550}) / (t \times (\text{VA}/\text{VR}) \times \text{OD}_{600}) \quad (3)$$

The values were then normalised to the activity of WT.

2.14. Growth assay

Strains were activated in liquid media overnight and diluted to 0.05 OD₆₀₀/ml with fresh culture media. The cell suspension was transferred onto a clear flat-bottomed 96-well microtiter plate (Thermo Fisher Scientific) at a volume of 130 µl/well. Measurements were taken at $\lambda=600$ nm every two-minute interval with continuous orbital shaking at 30°C using Tecan Infinite 200 microplate reader under the i-control software.

2.15. Spotting assay

Strains were activated in liquid media overnight and diluted to 0.2 OD₆₀₀/ml with fresh culture media, from which three 10-fold serial dilutions were prepared. The cell suspensions were then spotted on to appropriate agar plates and incubated in 30°C for 3 days.

2.16. Crude mitochondria isolation

Crude mitochondria isolation was carried out as previously described (Gregg, Kyryakov, & Titorenko, 2009). Briefly, 300 OD₆₀₀ of early log phase cells are washed with water and its wet weight measured.

The cells are resuspended in DTT buffer (100 mM Tris/H₂SO₄ pH9.4, 10 mM dithiothreitol) at 2 ml/g (wet weight of cells). Cells are incubated for 20 min at 30°C at 70 rpm and spun down at 3000 x g for 5 min, RT. The pellet was solubilized in zymolase buffer (20 mM potassium phosphate pH7.4, 1.2 M sorbitol) at 7 ml/g and incubated with 100T zymolase at 1 mg/ml at 30°C for 30 min. Spheroplasts were pelleted down at 2200 x g for 8 min, 4°C and resuspended in ice-cold homogenization buffer (10 mM Tris/HCL pH 7.4, 0.6 M sorbitol, 1 mM EDTA, 0.2% BSA) at 6.5 ml/g. Spheroplasts were homogenized by 15 strokes of the dounce homogenizer and the lysate clarified at 1500 x g for 5 min, 4°C. The supernatant is spun down at 3000 x g

for 5 min, 4°C followed by 12000 x g for 15 min, 4°C. The pellet is resuspended in 100 µl, lyophilized, and proceeded for lipid analysis.

Chapter 3. Lipid perturbation compromises UPR-mediated ER homeostasis as a result of premature degradation of transmembrane proteins

3.1. Introduction

Previous work from our lab revealed that cells respond to lipid perturbation through predominant compensatory changes to the proteome, with minimal changes to the lipidome (Thibault et al., 2012). Having the biggest impact under LP, changes in the proteomics of the cell were analysed to understand the impact of LP on the cell. Certain outliers were identified where the proteins were found to be in low abundance while their corresponding mRNA was found to be transcriptionally upregulated. It remains unclear whether the proteins were either downregulated post-transcriptionally, or they were degraded more rapidly and have shorter half-lives. This also raises the interesting question whether there is any common factor or reason which explains this phenomenon.

A large-scale approach should be taken to account for this global phenomenon, which affects multiple pathways and organelles in the cell. Hence, a screening of the protein candidates would allow us to common pathways or organelles which are being affected similarly under LP. *opi3Δ* was used as a model for LP instead of *cho2Δ* due to the mutant exhibiting a stronger phenotype of LP than *cho2Δ* (Thibault et al., 2012). PC/PE homeostasis in *opi3Δ* is more severely disrupted, and exhibited a stronger impact on cells than *cho2Δ*. Hence, it will allow us to identify significant subtle differences in cells under LP.

3.2. An *in silico* screen identified predominantly ER TPs as being targets for having decreased abundance despite being transcriptionally upregulated

We carried out *in silico* screening to identify proteins that were upregulated transcriptionally yet displayed significantly lower protein abundance under LP. In total, 66 proteins candidates were picked out; exhibiting an increase in RNA transcription, yet having a lower protein abundance of more than 2-fold change. An enrichment of transmembrane proteins was found in the proteins identified (Table S1). Among the 66 protein candidates, 27 (40%) of them were membrane proteins, which was higher than the estimate of 20-30% of proteins being membrane proteins (Figure 3.1) (Krogh, Larsson, von Heijne, & Sonnhammer, 2001). Among the membrane proteins, 11 proteins reside in the ER, indicating that a significant proportion (17%) of ER membrane resident proteins are being affected (Kumar et al., 2002). One plausible reason was that LP has a large impact on the biophysical properties of the ER membrane. The ER membrane composed of around 51% PC, and hence the drastic change in lipid composition of the ER membrane might have affected ER TPs. We focused on ER-resident proteins to rule out other effects of LP on the cell such as transport and secretion.

3.3. Cue1, Emc4, Nsg2 and Sbh1 were selected being part of the UPR programme

To confirm our previously published largescale data (Thibault et al., 2012), we analysed the steady-state level of ten TPs candidates under LP using PC synthesis deficient strain *opi3Δ* under their endogenous promoters (Figure 3.2). Six TPs, Ctr1, Nsg2, Prm5, Sbh1, Scs7 and Yet3, exhibited significant changes in their protein steady state levels in *opi3Δ* as compared to WT. Ctr1, Nsg2, Sbh1 and Scs7 had significantly lower steady-state levels. Surprising, Prm5 and Yet3 exhibited higher protein steady-states. Overall, the steady-state levels of the TPs analysed are lower during LP.

Figure 3.1. ER resident membrane proteins form a large fraction of proteins having decreased abundance despite being transcriptionally upregulated.

Out of 66 proteins, 11 (17%) have decreased abundance despite being transcriptionally upregulated are ER resident proteins. Localisation of the proteins were obtained from *Saccharomyces* Genome Database (SGD).

Figure 3.2. Certain TPs are verified to have low abundance under LP.

Steady state level of transmembrane proteins. Equal cell numbers were harvested. Proteins were separated by SDS-PAGE and detected by immunoblotting with antibodies against the HA tag and Tub1 as loading control. The expected protein levels are shown in brackets. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, Student's t test.

We confirmed that the largescale data reasonably provided TPs candidates that are upregulated transcriptionally but have lower protein abundance. We selected Cue1, Emc4, Nsg2 and Sbh1 to proceed with our investigation as they are part of the UPR

programme and play important roles in ER homeostasis. Cue1 is an ubiquitin-binding protein that recruits the ubiquitin-conjugating enzyme Ubc7 to the ER for the degradation of misfolded proteins via the ERAD pathway (Figure 1.6) (Biederer, Volkwein, & Sommer, 1997). ERAD is critical under ER stress in eliminating misfolded proteins in the ER and preventing their accumulation (Vembar & Brodsky, 2008). Emc4 is a member of the ER transmembrane complex (EMC). EMC plays a critical role in protein folding at the ER but the exact role of the protein in the complex remains unclear (Jonikas et al., 2009). Protein folding processes are essential to help newly synthesised proteins achieve their desired three-dimensional configurations for their functions. Nsg2 is involved in the regulation of Hmg2, an isoenzyme of HMG-CoA which catalyses the rate-limiting step in sterol biosynthesis (Flury et al., 2005). The synthesis of ergosterol is essential to maintain both membrane structural integrity and fluidity in yeast. Homeoviscous adaptation carried out by ergosterol aids cells in restoring membrane fluidity, buffering against adverse changes in membrane fluidity. Sbh1 is the highly conserved β subunit of Sec61 ER translocation complex and is involved in the translocation of proteins into the ER (Panzner, Dreier, Hartmann, Kostka, & Rapoport, 1995). Protein translocation into the ER is the first crucial step in the secretory pathway, while retro-translocation is the process by which misfolded proteins are removed from the ER back into the cytosol for degradation by the 26S proteasome. Together, translocation and ERAD act in a concerted fashion to reduce accumulation of misfolded proteins in the ER (Chevet, Cameron, Pelletier, Thomas, & Bergeron, 2001; Romisch, 1999; E. Spear & Ng, 2001).

Cue1, Emc4, Nsg2 and Sbh1 play important roles in downstream UPR response to alleviate ER stress (Biederer et al., 1997; Flury et al., 2005; Jonikas et al., 2009; E. Spear & Ng, 2001). We expected these proteins to be present at higher levels under lipid disequilibrium like their corresponding mRNA. However, the actual decrease in

steady states or the failure to be misplaced with these proteins indicate that they might be translationally downregulated or destabilised.

3.4. TPs are upregulated transcriptionally

To validate the DNA microarray results of these 4 TP candidates, quantitative real-time PCR (qPCR) was performed (Figure 3.3). *opi3Δ* cells were used as the sole representative of LP as they exhibit a stronger phenotype on the destabilisation of the TPs than *cho2Δ*. *CUE1*, *EMC4*, *NSG2* and *SBH1*'s mRNA of *opi3Δ* and WT were found to be upregulated and similar to the levels determined by DNA microarray (Figure S1).

The upregulation in the mRNA levels of the subset of TPs will normally lead to the translation of more TPs. However, protein analyses have shown that the general steady state levels of the subset of TPs were lower under LP (Figure 3.2). Hence, we investigated whether the decrease seen is due to a decrease in half-lives of the TPs.

3.5. TPs are prematurely degraded under LP

To assess the stability of TP candidates during lipid imbalance, cycloheximide chase assay was performed in WT and *opi3Δ* strains. Cycloheximide blocks translation by binding to the ribosomes and interferes with the elongation of polypeptides (Baliga, Pronczuk, & Munro, 1969). Time points were taken from adding cycloheximide to measure the half-lives of the tagged-proteins over a period of one hour. Pgk1 serves as a loading control to normalise the levels of proteins detected in the gels.

Cue1, Emc4, Nsg2 and Sbh1's half-lives were found to be reduced under LP (Figure 3.4). A small but significant decrease of 14% in Cue1-HA protein level was detected in Δ *opi3*. A moderate decrease of 27% in protein levels of Emc4-HA was found in *opi3Δ*. For Nsg2, a large decrease of 41% in protein levels was found in *opi3Δ*. Sbh1 exhibits the largest decrease in protein level under lipid disequilibrium; 58% for *opi3Δ*. The TPs

were found to have lower protein levels under LP, indicating that the TPs were being degraded at a faster rate. This supports the idea that while being transcriptionally upregulated, the TPs are being degraded prematurely.

Figure 3.3. Validation of DNA microarray analysis by qPCR.

WT and *opi3Δ* cells were grown to early log phase at 30°C in synthetic complete media. Total RNA was then harvested and the expression levels of *CUE1*, *EMC4*, *NSG2* and *SBH1* genes were assessed by quantitative real time PCR (qPCR). Based 2 logarithmic fold changes in mRNA levels in *opi3Δ* were normalised to WT levels. Error bars show the SD of three technical replicates.

3.6. TPs remains localised on the ER membrane during LP

To ensure that Cue1, Emc4, Nsg2, and Sbh1 are not prematurely degraded due to mislocalisation, indirect immunofluorescence was performed with the four candidate proteins in WT and *opi3Δ* cells. We introduced the TPs under the control of their own endogenous promoter as well as under the expression promoter *PGK1*. The TPs were found to co-localise with the molecular chaperone ER marker Kar2 in *opi3Δ* cells in both endogenous as well as overexpressed expression levels (Figure 3.5 A and B respectively).

Figure 3.4. TPs are prematurely degraded under LP.

Degradation of HA-tagged proteins was analysed after blocking protein translation with cycloheximide. Proteins were separated by SDS-PAGE and detected by immunoblotting with antibodies against the HA tag and Pgk1 as loading control.

Figure 3.5. TPs remain localised on the ER transmembrane under LP.

Cells expressing TPs endogenously (A) as well as under *PGK1* promoter (B) were grown to early log phase at 30°C in selective synthetic complete media before being fixed by formaldehyde and permeabilized. TPs tagged with the HA tag were detected using anti-HA antibody and anti-Kar2 was used to stain the nuclear envelope/ER membranes. Scale bar, 5 μm.

3.7. TPs remains integrated on the ER membrane during LP

We further investigated that the TPs being integral membrane proteins, remain embedded and integrated into the ER membrane. Alkaline carbonate extraction was performed to disrupt membranes, and associated proteins and soluble contents of the cell are separated from the membranes. Only integrated proteins will remain associated to membranes and be recovered after ultracentrifugation. The destabilised TPs was found in same fraction as the TP Sec61, confirming that the TPs remain integrated into the ER membrane, while not being detected in the same fraction as the soluble protein Kar2 (Figure 3.6).

Figure 3.6. TPs remain integrated into the ER transmembrane under LP.

Microsome membranes prepared from wild type and *opi3Δ* expressing HA tagged proteins were treated with 0.1 M sodium carbonate, pH 11, for 30 min on ice. A portion was reserved as total (T), and the remaining was subjected to centrifugation at 100,000 x g. Supernatant (S) and membrane pellet (P) fractions were collected and analysed by immunoblotting. Proteins were detected using anti-HA antibody. Kar2 and Sec61 serve as soluble and integral membrane protein controls, respectively.

3.8. TPs topology remains unaffected under LP

One plausible reason for the rapid degradation of these TPs is that they might be inserted inversely into the ER membrane, or flipping of the TPs might occur under LP. This will cause the proteins to be recognised as foreign or misfolded entities, resulting in their rapid degradation. To ensure the premature degradation of these four proteins is not due to inverted topology, we performed proteinase K (PK) digestion from isolated microsomes. In WT cells, C-termini HA tags of Cue1-HA, Emc4-HA and Nsg2-HA are found to be in the cytosol (Figure 3.7 A). Thus, HA tag will be cleaved off if the topology is intact while a peptide will be detected if the topology is inverted upon PK digestion. Sbh1-HA is a tail-anchored TP where the C-terminal HA tag of Sbh1-HA is found in the ER lumen and hence will remain to be detected under intact topology.

The three proteins were found to be fully digested under LP and the predicted smaller protein fragments of 23.7, 6.9, and 5.7 kDa were not detected for Cue1-HA, Emc4-HA, and Nsg2-HA, respectively, in both WT and *opi3Δ* (Figure 3.7 B). The predicted protein fragment of 9.5 kDa in Sbh1 was detected in both WT and *opi3Δ* strains. Together, these results suggest that the four TP topologies are not affected from LP and thus eliminating this factor as an underlying cause for their premature degradation.

3.9. TPs are stabilised by restored PC levels under LP

We hypothesized that the low levels of PC found in *opi3Δ* cells decreased the stability of the candidate proteins (Figure 3.4). To confirm the four candidate proteins are destabilised from low PC levels, their degradation was monitored in the presence of choline. Choline is rapidly converted into PC through the Kennedy pathway to restore PC level of *opi3Δ* to be similar to WT (Figure 1.3) (Carman & Henry, 1989; Thibault et al., 2012). Choline supplementation in *opi3Δ* was sufficient to fully stabilise Cue1-HA, Emc4-HA, Nsg2-HA, and Sbh1-HA to the levels found in WT (Figure 3.8).

Figure 3.7. TPs' topology remains unaffected under LP.

Microsomes membranes prepared from WT and *opi3Δ* cells expressing HA tagged proteins were treated with 1 mg/ml proteinase K, for 30 min at 37°C, with or without 1% NP40. HA-tagged were precipitated with 10% TCA, separated by SDS-PAGE and detected by immunoblotting with HA antibody. Expected proteins molecular weight are shown below for non-digested (N), digested (D), and flipped and digested (F-D). HA tag is shown as black dot. Fragments missing HA tag are not detectable and are illustrated with transparency. ER lumen and cytosol are at the top and bottom of the membrane, respectively.

3.10. Sbh1 was selected as a model for the destabilised ER TPs

To further understand how TPs are destabilised under LP, Sbh1 was selected as a model substrate. In our previous study (Thibault et al., 2012), a translocation defect was found to occur in cells under LP. From that proteomic analysis, only Sbh1 in the co-translational and post-translational complex (Figure 1.7) is found to have decreased stability in both *cho2Δ* and *opi3Δ* calls as compared to WT cells. Under WT conditions, Sbh1 was found to be non-essential for translocation (Finke et al., 1996). As only Sbh1 is prematurely degraded under LP, we wondered whether Sbh1 might be essential for translocation under LP, and the translocation defect seen is due to the premature degradation of Sbh1. Translocation into the ER is the first step in the secretory pathway, where a delay in translocation might result in a bottleneck which further

delays the entire pathway. Hence, we decided to proceed our investigations with Sbh1 as a model substrate to further investigate the destabilisation of the ER TPs.

3.11. UPR activation did not destabilise Sbh1

Under ER stress, the UPR is activated to regulate ER protein synthesis and quality control. The UPR is strongly activated under LP to alleviate ER stress (Jonikas et al., 2009; Thibault et al., 2012). As the UPR broadly changes the proteomic landscape of the cell, we wanted to verify that Sbh1 is not destabilised from UPR activation. We introduced a constitutive active form of the downstream effector, Hac1ⁱ, into WT (Chapman & Walter, 1997; Thibault et al., 2011), and monitored the degradation of Sbh1. Constitutively activated UPR did not destabilise Sbh1 in WT cells (Figure 3.9). This eliminates the possibility that Sbh1 was destabilised from a strong UPR response instead of by LP. A higher protein steady state was also seen from the zero-time point in WT expressing *HAC1i* as compared to WT alone (2.7 fold higher). This was expected as *SBH1* is upregulated from the UPR programme (Thibault et al., 2012; Travers et al., 2000). Thus, this indicates that the UPR activation in *opi3Δ* is not a contributor to Sbh1 premature degradation.

Figure 3.8. Choline supplementation stabilises TPs under LP.

Cells were grown in the absence or presence of 1 mM choline before addition of cycloheximide. Time points were taken as indicated. Proteins were separated by SDS-PAGE and detected by immunoblotting with antibodies against HA tag and Tub1 as loading control.

Figure 3.9. Activation of UPR did not cause the premature degradation of Sbh1.

The degradation of Sbh1-HA was analysed in WT and *opi3Δ* cells containing control vector (ve) or *HAC1i*-bearing plasmid after blocking translation with cycloheximide. Proteins were separated by SDS-PAGE and detected by immunoblotting with antibodies against HA tag and Tub1 as loading control.

3.12. Cells shortens their fatty acid chain lengths in the ER under LP

As the TPs remain integrated in the ER membrane with their topologies intact and the supplementation of PC might restore back their stabilities, we explored whether there are changes in the lipid composition of the ER membrane under LP. We saw an enrichment of ER TPs that exhibited an upregulation in mRNA but a decrease in protein abundance (Figure 3.1). Hence, we analysed the fatty acid (FA) composition of whole cells and fractionated microsomes from WT and *opi3Δ* to characterise lipid remodelling specific changes to the ER in *opi3Δ* that might contribute to the premature degradation of TPs. Overall, total FAs in *opi3Δ* cells increased by 96% when compared to WT (Figure 3.10 A). Increased total FA composition might occur as *opi3Δ* accumulates large lipid droplets (Fei et al., 2011; Thibault et al., 2012). From isolated microsomes, a large increase of the short FAs C14:0 and C14:1 and a decrease of C18:1 were observed in *opi3Δ* when compared to WT (Figure 3.10 A, B). It was also apparent that lipid remodelling by the shortening of FA chains is more drastic in *opi3Δ* microsomes compared to whole cells.

It was previously reported that the intermediate for the synthesis of PC from PE, MMPE, becomes the most abundant phospholipid with the knockout of the *OPI3* gene (Thibault et al., 2012). As MMPE shared similarities with PE by just the addition of one methyl group, a large increase in the ER membrane composition is expected to induce negative membrane curvature stress as previously reported for PE (J. E. Vance & Tasseva, 2013). This is also supported in that PC might be substituted by DMPE, in the *de novo* pathway in certain scenarios for survival but not with MMPE (McGraw & Henry, 1989). We speculated that the shortening of FAs might be an adaptive response to alleviate membrane curvature stress in *opi3Δ* (Zimmerberg & Kozlov, 2006). In addition to the shortening of FAs, we investigated for changes in the saturation state of the FA, as another remodelling to adapt to membrane curvature stress. To investigate for changes in saturation states of FA, we introduced the stearoyl-CoA desaturase gene *OLE1* under the control of the expression promoter *PRC1* to increase monounsaturated fatty acids (MUFAs) to saturated fatty acids to elevate membrane fluidity (Stukey, McDonough, & Martin, 1989). As *OLE1* is an essential gene, the knockout of *OLE1* might not be used to mimic the decrease of membrane fluidity found in *opi3Δ*. We verified that there is a decrease in the saturation of FAs by 1.9% from the overexpression of *OLE1* (Figure 3.11). Under LP, we detect a significant increase of 4.3% of saturated FA. The overexpression of *OLE1* in cells under LP is also able to reduce the saturation of the FAs in the ER.

Figure 3.10. Shortening of FA in ER membrane as well as whole cells occur under LP.

Total fatty acid content in whole cells and microsomes of WT and *opi3Δ* were quantified by gas chromatography after FAME derivatisation. Fold changed (FC) of FAs in *opi3Δ* is compared to WT in whole cells and microsomes (A). Percentage of fatty acid chain lengths to total fatty acids in *opi3Δ* is compared to WT in microsomes (B).

Figure 3.11. Increase in saturation of FA in ER membrane occur under LP.

Percentage of saturated fatty acids to total FAs in WT and *opi3Δ* under endogenous and overexpression (OE) of *OLE1*. OE of *OLE1* is under the control of the *PRC1* promoter. Total fatty acid content in microsomes of WT and *opi3Δ* were quantified by gas chromatography after FAME derivatisation. Student's t test compared to WT.

3.13. ER membrane experiences a decrease in membrane fluidity under LP

FA saturation states of biological membranes are highly linked to membrane fluidity (Quinn, 1981; Stubbs, 1983; Stubbs & Smith, 1984). To better understand the impact of membrane remodelling on TPs mobility, we monitored the dynamic of the ER-resident TP Sec63-GFP by fluorescence recovery after photobleaching (FRAP) (Shibata et al., 2008). A region of the cortical ER is photobleached and its fluorescence recovery is quantified which correlates with the mobility of Sec63-GFP. The mobility of Sec63-GFP, an ER resident protein, provides an indication for the relative mobility of the ER membrane (Tank, Wu, & Webb, 1982; Wey, Cone, & Edidin, 1981).

The fluorescence recovery of Sec63-GFP was significantly slower in *opi3Δ* compared to WT suggesting the ER membrane might be stiffer (Figure 3.12 A, B). This result is consistent with the reported observations that decreased PC/PE ratios stiffen the membranes (Dawaliby et al., 2016). With the overexpression of *OLE1*, we detected a small but significant increase in fluorescence recovery. However, we detected no recompensation of recovery in *opi3Δ* with the overexpression of *OLE1*, where recovery of the strain remains similar to *opi3Δ*. Even though the saturation of the acyl chain decreases (Figure 3.14), there is no corresponding increase in Sec63-GFP mobility. This might be due to other factors such as protein-lipid interactions, increased in membrane proteins that have occurred under LP to affect the protein mobility in the ER membrane.

Figure 3.12. A decrease in Sec63-GFP mobility occur under LP.

Fluorescent recovery of Sec63-GFP in WT and *opi3Δ* under endogenous and overexpression of *OLE1* after photobleaching. A region of the cortical ER of live cells were photobleached and recovery points of 1.5 s interval were taken. Fluorescence recovery after photobleaching (FRAP) is shown for the first 60 seconds from the average of 20 cells (A). The time taken for the recovery of half the maximum fluorescent ($t_{1/2}$) was calculated and plotted (B). Student's t test compared to WT.

3.14. Sbh1 is destabilised from decreased membrane fluidity of the ER membrane

We proceeded to investigate the role of membrane fluidity on the stability of Sbh1. The overexpression of *OLE1* which will result in an increase in MUFAs resulted in the rapid degradation of Sbh1 in WT cells like the degradation rate observed in *opi3Δ* (Figure 3.16). Without the restoration of membrane fluidity (Figure 3.12), Sbh1 remains destabilised in *opi3Δ* even with the overexpression of *OLE1*.

The change in membrane fluidity solely is able to induce the premature degradation of Sbh1. Interestingly, bidirectional changes in the ER membrane fluidity both exhibited the destabilisation of Sbh1. This suggests that certain TPs might be more sensitive to changes in membrane fluidity, where the loss in the homeostasis of membrane fluidity

might result in their premature degradation. The ER membrane might also be highly affected unlike plasma membrane, where the increased presence of ergosterol might stabilise the membrane.

Figure 3.13. Sbh1 becomes destabilised from a decrease in membrane fluidity.

The degradation of Sbh1-HA in WT and *opi3Δ* cells was analysed in the absence (control vector) or presence of OE *OLE1* after blocking translation with cycloheximide. Proteins were separated by SDS-PAGE and detected by immunoblotting with antibodies against HA tag and Tub1 as loading control.

3.15. Discussion

Chronic metabolic pathologies such as obesity, diabetes, liver disease and cardiovascular disease represent the greatest global public health problem today especially in developed countries (Arruda & Hotamisligil, 2015; Khaw, Choi, Kam, Chakraborty, & Chow, 2016; Leamy et al., 2013). Novel solutions are in great demand to fight the rising health care cost from the onset of such pathologies. While advances are made in the understanding of the development of chronic diseases, the fundamental principles behind how cells fail to maintain homeostasis under LP remains elusive. In this chapter, we seek to explore one of the possible mechanisms in which cells fail to alleviate ER stress, resulting in the onset for the development of diseases.

We attributed the perturbation in the balance of the two most abundant phospholipids PC and PE to the cause of the destabilisation of the ER TPs. We detected an enrichment of ER TPs that have low abundance despite being transcriptionally upregulated under LP (Figure 3.1). This might be due to the low PC abundance found in the ER membrane (Figure 1.2), as well as the low presence of ergosterol that might help buffer against changes in the biophysical properties of the ER membrane (Abe & Hiraki, 2009). This might make the ER more susceptible to membrane fluidity changes that occurred under LP as proposed in this study.

We have ruled out that mislocalisation of the TPs are the cause of their destabilisation (Figure 3.5), and the TPs are integrated into the ER membrane (Figure 3.6). Insertion of the TPs into the ER membrane are also unaffected (Figure 3.7), and restoration of PC synthesis fully stabilises the TPs (Figure 3.8). The constitutive activation of the UPR did not have a negative impact on the stability of Sbh1 (Figure 3.9). Taken together, these results provide strong evidences that the TPs are destabilised from changes in the environmental lipid composition of the ER membrane.

We sought to understand the changes in the biophysical properties of the ER membrane which cause the destabilisation of the TPs. It is largely reported that PC has a cylindrical shape with a cross-sectional area for the head group similar to the acyl chain tails, generating minimal curvature and forming flat lamellar phase phospholipid bilayer (Szule, Fuller, & Rand, 2002). PE is classified as cone-shaped lipid which is known to form non-lamellar membrane structure as it generates negative membrane curvature (J. E. Vance & Tasseva, 2013). MMPE become highly abundant under the ablation of *opi3Δ*, and being mono-methylated, has physical properties similar to PE. The increase in membrane curvature from the replacement of PC to MMPE might have induced cells under LP to remodel and decrease their fatty acid chain lengths

according to the seminal Helfrich theory of membrane bending elasticity (Figure 3.10) (Zimmerberg & Kozlov, 2006). A larger remodelling of the chain length seen in the ER as compared to in the whole cells suggests either the ER is more susceptible to LP due to high PC content in the ER membrane, the minimal presence of ergosterol to regulate the membrane bilayer in the ER (Zinser, Paltauf, & Daum, 1993), or the cells respond more aggressively to the ER membrane bilayer to alleviate ER stress. Accordingly, an increase in lipid packing from the increase in saturation state will also allow lesser curvature to be induced (Figure 3.11). Additionally, PC is largely reported to be highly unsaturated (Holthuis & Menon, 2014; Pineau et al., 2008), and hence its absence might further exacerbate a decrease in saturation.

FAs remodelling might be an adaptive response to alleviate membrane curvature stress in *opi3Δ*. Both shortening and increased saturation of FAs will serve to reduce negative membrane curvature stress from the abundance of MMPE under LP (Zimmerberg & Kozlov, 2006). In a separate study, the depletion of PC in *cho2Δopi3Δ* was also found to increase lipid acyl chain saturation as well the shortening of the acyl chains in the lipidome of whole cells (Boumann et al., 2006). Changes in the composition of acyl chain are often made to adjust the thickness, curvature or fluidity of the membrane bilayer. The coupling of shortening with increased saturation is not compatible with the unidirectional changes in membrane thickness or fluidity. For example, an increase in membrane fluidity will result in increased saturation together with the lengthening of the acyl chain composition as membrane adaptation. Shortening of the acyl chains might result from the early release of newly synthesised fatty acids from the fatty acid synthase. Alternatively, it might also be from the reduction in activity of elongases Elo1 and/or Elo2 (Rossler, Rieck, Delong, Hoja, & Schweizer, 2003; Schneiter, Tatzer, Gogg, Leitner, & Kohlwein, 2000). For the

increased in acyl chain saturation, the decreased in Ole1 activity might have attributed to the change, as it is the only fatty acid desaturase in yeast.

The remodelling of the ER membrane to alleviate negative membrane curvature stress might have imposed further challenges to cells under LP. An increase in saturation of the fatty acid chain will have impacted the membrane bilayer to be less fluid, and we made similar observations (Figure 3.12). With a low proportion of ergosterol present in the ER membrane bilayer, such changes in saturation state of the ER might have a large impact on membrane fluidity. Similarly, the replacement of PC with MMPE in cells under LP will also result in a decrease in membrane fluidity (Dawaliby et al., 2016). We further verified that a change in membrane fluidity might destabilise our model TP, Sbh1 (Figure 3.13).

We investigated whether the mobility of transmembrane proteins (TPs) would be affected by changes in lipid composition, and propose that membrane is stiffer under LP. The decrease of PC in liposomes makes the membrane stiffer, indicating LP could also result in the stiffening of ER membrane (Dawaliby et al., 2016). However, investigating Sec63-GFP mobility through FRAP might not be a good proxy for estimating membrane fluidity. TPs mobility in the ER may be affected by other factors such as changes in protein modifications and interactions experienced by the TPs independent of membrane fluidity. Hence, additional experiments that directly measure membrane fluidity could further verify the stiffening of the ER membrane under LP. The usage of dyes that interact with phospholipids such as Laurdan can allow us to further investigate the fluidity of the ER membrane (Sanchez, Tricerri, & Gratton, 2012). However, membrane properties might be affected from the addition of the dye. Therefore, stiffening of ER membranes under LP can be further validated to support our hypothesis.

In conclusion, we proposed a model where LP might have compromised the activation of the UPR (Figure 3.14). Restoration of PC/PE homeostasis might fail and perturbed PC/PE have been found in obese mouse models (Fu et al., 2011). We have modelled this LP using *opi3Δ* mutant cells in yeast. Our LP models have amplified the PC/PE imbalance to see the effects of LP, and how cells respond to LP. The UPR is activated as a result to alleviate ER stress (Thibault et al., 2012). The downstream response of the UPR includes the transcription of TPs situated on the ER membrane involved in alleviating ER stress (Travers et al., 2000). While being transcriptionally upregulated, they were prematurely degraded on the protein levels (Figure 3.7). We deduced that after lipid remodelling of the ER, the fluidity of the ER membrane becomes low (Figure 3.12), resulting in the destabilisation of certain ER-localised TPs (Figure 3.4). Additionally, the absence of the rich unsaturated fatty acid provider, PC, might also contributed to the stiffening of the membrane (Dawaliby et al., 2016). Thus, these changes combined might have inevitably result in the loss of stability of certain TPs which are more sensitive to membrane fluidity changes. Being part of the UPR programme, the premature degradation of these TPs might result in a less effective response mounted to alleviate ER stress. With the restoration of ER homeostasis being hindered, it might eventually result in the development of chronic ER stress and the development of diseased states such as NALFD.

Figure 3.14. Premature degradation of TPs might lead to chronic ER stress and development of NAFLD.

Normally, ER homeostasis can be reached from lipid perturbation through the regulation of downstream UPR target genes. UPR transactivator (yellow protein representing Ire1, PERK, or ATF6) senses ER stress from the accumulation of misfolded proteins and/or lipid perturbation. However, if LP is prolonged, ER homeostasis might not be reached due to the premature degradation of a subset of misfolded proteins (blue protein) leading to chronic ER stress, cell death, and eventually the development of NAFLD.

Chapter 4. Sbh1 becomes dissociated from the Sec61 complex and is degraded by an exposed cytosolic motif under LP by ERAD

4.1. Introduction

In the previous chapter, we have identified certain ER TPs that were found to be destabilised under LP, and have found that changes in the biophysical properties of the ER membrane have destabilised Sbh1. We continued using Sbh1 to understand how it becomes destabilised under LP. In this chapter, we sought to understand the mechanism in which Sbh1 becomes recognised for degradation, as well as identify the consequences of its premature degradation under LP.

4.2. Sbh1 dissociates from the Sec61 complex under lipid imbalance

In the search for these answers, we began the investigation with a screening of the Sbh1 interactors. We performed the split ubiquitin based membrane yeast two hybrid (MYTH) assay in WT and *opi3Δ* cells to identify changes in Sbh1 membrane protein interactome (Paumi et al., 2007; Snider, Kittanakom, Damjanovic, et al., 2010). The bait construct was added at the N-terminus of Sbh1. The reverse tagging at the C-terminal was not carried out as luminal bait is not compatible with the assay. Localisation and expression levels of tagged Sbh1 were unaffected from this construct and remain similar to our previous experiments with WT and *opi3Δ*. The Sbh1 bait strain was transformed with a yeast genomic plasmid library in which ORF are fused to sequences encoding the prey sequence (Snider, Kittanakom, Curak, & Stagljar, 2010).

A total of 49 and 14 putative Sbh1-interacting proteins were identified in WT and *opi3Δ*, respectively. To eliminate false positive interactors, we performed the bait dependency test using the single-pass transmembrane domain of human T-cell surface glycoprotein

CD4 tagged to Cub-LexA-VP16 MYTH (Snider, Kittanakom, Curak, et al., 2010). CD4 serves as a negative control to ensure the preys identified are not ubiquitous in their interactions. In WT, we identified 38 proteins interactors of Sbh1 including previously reported interactors Ost4, Sec61, Ssa2, Ssb1, Sss1, and Yop1 (Figure 4.1 A; (Babu et al., 2012; Chavan, Yan, & Lennarz, 2005; Panzner et al., 1995; Zhao & Jantti, 2009)). Sbh1 was also found to exhibit interaction with membrane proteins involved in sterol biogenesis (Erg4, Erg24 and Nsg1) and fatty acid elongation (Elo2 and Tsc13). On the other hand, only 13 proteins were found to interact with Sbh1 in *opi3Δ* cells (Figure 4.1 B). Surprisingly, we did not detect an interaction of Sbh1 with Sec61 and Sss1, which together forms the translocation complex, in *opi3Δ*. Although the steady-state level of Sbh1 in *opi3Δ* is significantly reduced (Figure 3.2), the MYTH assay is relatively sensitive and transient interactors should be detected by the MYTH assay (Snider, Kittanakom, Damjanovic, et al., 2010). Thus, failure of Sbh1 to associate to the Sec61 complex under LP might be the cause for its premature degradation. This is consistent with the finding that Sbh2, the homologue of Sbh1, becomes destabilised and degraded rapidly when unbound to the Sec61-like complex Ssh1 (Habeck, Ebner, Shimada-Kreft, & Kreft, 2015).

Figure 4.1. Sbh1 dissociates from the Sec61 under lipid imbalance.

Proteins identified as interacting protein partners of N-tagged (TF-Cub-Sbh1) by the MYTH method in WT (A) and *opi3Δ* (B) cells. ERAD factors only detected in *opi3Δ* are denoted in red. Previously reported interactors of Sbh1 are indicated with black dots.

Interestingly, Sbh1 was found to interact with proteins of the ERAD pathway under LP (Figure 4.1 B). Sbh1 interactors include the membrane-embedded ubiquitin-protein ligase Doa10 parts of the ERAD Doa10 complex (Carvalho et al., 2006; Deng & Hochstrasser, 2006). As Doa10 complex is generally specific for substrates containing cytosolic lesions (ERAD-C) (Vashist & Ng, 2004), it suggests that a polypeptide stretch of Sbh1 might become exposed on its cytosolic side under lipid disequilibrium making it susceptible to ubiquitination. Subsequently, targeted substrates for degradation are polyubiquitinated in the cytosol by the addition of Lys-11-linked ubiquitin (Ubi4) identified to interact with Sbh1 in *opi3Δ* cells. The AAA⁺ ATPase protein Cdc48 was also found to interact with Sbh1 in *opi3Δ* cells (Figure 4.1 B). Ubiquitinated substrates are retro-translocated to the cytosol by the action of the Cdc48 complex and targeted to the proteasome for degradation (Braun, Matuschewski, Rape, Thoms, & Jentsch, 2002; Ye, Meyer, & Rapoport, 2001). Another important player of the ERAD pathway,

Png1, was found to exclusively interact with Sbh1 under lipid imbalance. Png1 catalyses the deglycosylation of misfolded glycoproteins, and is a critical step for ERAD substrates before degradation (Suzuki, Park, Hollingsworth, Sternglanz, & Lennarz, 2000). Together, the MYTH screening results might suggest the dissociation of Sbh1 from the Sec61 complex, resulting in its rapid degradation through the ERAD pathway.

4.3. Sec61 and Sss1 remain stable under LP

We carried out cycloheximide chase assay to follow the stability of Sec61-Flag and Sss1-Flag. Both Sec61 and Sss1 were found to be stable in *opi3Δ* as in WT in agreement with our previously reported proteomic data (Figure 4.2) (Thibault et al., 2012). This rule out the possibility that Sbh1 became destabilised indirectly from the loss of stability of its interacting partners.

4.4. Sbh1 can still interact with the Sec61 complex under LP

We moved on to further access if interaction between Sbh1 with the Sec61 complex on the ER membrane is possible under LP. To capture this interaction *in vivo*, proteins were cross-linked before carrying out co-immunoprecipitation. Proteins in both WT and *opi3Δ* cells were treated with the cross-linker Dimethyl 3,3'-dithiobispropionimidate (DTBP) before the pull down of Sbh1-HA. DTBP stabilises protein interactions prior to co-immunoprecipitation (Co-IP) assay to capture protein interactions before the proteins were extracted. Sec61 was found to be co-immunoprecipitated by Sbh1-HA in both WT and *opi3Δ*, verifying that Sbh1 can still interact with the translocon under lipid perturbation (Figure 4.3). This justifies that Sbh1 ability to interact with Sec61 complex is not compromised under LP. However, changes in the ER membrane lead to the loss of interaction between Sbh1 with the Sec61 complex.

Figure 4.2. Sec61 and Sss1 are stable under LP.

The degradation of Sec61 or Sss1-Flag was analysed in WT and *opi3Δ* cells after blocking translation with cycloheximide. Proteins were separated by SDS-PAGE and detected by immunoblotting with antibodies against Sec61 or Flag tag and Tub1 as loading control.

Figure 4.3. Sbh1 can still interact with Sec61 under LP.

Immunoprecipitation of Sbh1-HA with protein G beads were analysed in WT and *opi3Δ* cells after proteins are cross-linked by DTBP. Elution and input samples were resolved by SDS-PAGE, transferred to nitrocellulose, and analysed by immunoblot with antibodies against Sec61 and the HA tag after the release of HA bound Sbh1 by HA peptide.

4.5. Sbh1 is degraded through ERAD under LP

We saw ERAD factors as interactors of Sbh1 under LP (Figure 4.1 B). This suggests that Sbh1 can be recognised and rapidly degraded under LP via the ERAD degradation pathway. We also wanted to investigate whether Sbh1 can be dependent on the vacuolar pathway for degradation. The vacuolar pathway serves as an alternate means for degradation of misfolded proteins in ER. While most ER proteins are degraded by ERAD, some proteins are packaged into vesicles and transported to the vacuole for degradation. To determine whether Sbh1 is dependent on the ERAD or vacuolar pathway for degradation, we investigated the degradation of Sbh1 in *cue1Δopi3Δ* and *pep4Δopi3Δ*. Cue1 is essential to target Ubc7, a E2 ubiquitin-conjugating ligase for the ERAD complex, and absence of Cue1 results in the accumulation of ERAD substrate at the ER (Biederer et al., 1997). We genetically inhibit the vacuolar protein degradation using *pep4Δ*. Proteinase A encoded by the *PEP4* gene is involved in the activation and maturation of various downstream vacuolar enzymes in the vacuolar pathway, directly cleaving other major proteases, namely carboxypeptidase Y, Proteinase B and aminopeptidase I (Ammerer et al., 1986; Parr, Keates, Bryksa, Ogawa, & Yada, 2007). The absence of proteinase A results in the accumulation of vacuolar substrate at the vacuole.

Sbh1 was shown to be stabilised in the *opi3Δcue1Δ* mutant, showing dependency on ERAD for degradation (Figure 4.4). Sbh1 was not fully stabilised like WT, but it can be explained that the accumulation of Sbh1 over time can lead to bulk degradation by the alternate vacuolar pathway. In *opi3Δpep4Δ* mutant, degradation of Sbh1 remains similarly to *Δopi3* where blocking the vacuolar degradation pathway did not stabilise the protein. This indicates ERAD as the recognised degradation mechanism for Sbh1 under lipid disequilibrium.

Figure 4.4. Sbhl1 degradation is mediated by the ERAD pathway under lipid disequilibrium.

The degradation of Sbhl1-HA was analysed in WT, *opi3Δ*, *opi3Δcue1Δ*, and *opi3Δpep4Δ* cells after blocking translation with cycloheximide. Proteins were separated by SDS-PAGE and detected by immunoblotting with antibodies against the HA tag and Pgk1 as loading control.

4.6. Sbhl1 is degraded through ERAD by the Doa10 complex

We have also identified the E3 ligase Doa10 among the interactors of Sbhl1 under LP (Figure 4.1 B). ERAD is carried out by two complexes, known as the Doa10 complex and the Hrd1 complex (Figure 1.6). The Doa10 complex recognises misfolded protein from their cytosolic lesions (ERAD-C), while Hrd1 complex recognises misfolded proteins from their luminal (ERAD-L) and transmembrane lesions (ERAD-M). Hrd1 forms a 1:1 stoichiometric complex with Hrd3, where the complex is sufficient for the ERAD-M degradation. Usa1 is a transmembrane protein with a large cytosolic domain and functions as a linker that bridges Der1 to the Hrd1/Hrd3 core complex. It is required for ERAD-L, but is not required for ERAD-M (Ismail & Ng, 2006). To further validate that Sbhl1 is degraded in a Doa10-dependent manner, we carried out cycloheximide chase assay to monitor Sbhl1 stability in these different ERAD mutants. Sbhl1 was found to be fully stabilised in *opi3Δdoa10Δ* but not in *opi3Δhrd1Δ* and

opi3Δusa1Δ mutants (Figure 4.5). Together with MYTH data, it suggests that Sbh1 is exclusively targeted for degradation by the ERAD *doa10* complex through a cytosolic lesion.

Figure 4.5. Sbh1 is recognised by ERAD from its cytoplasmic domain under lipid imbalance.

The degradation of Sbh1-HA was analysed in WT, *opi3Δ*, *opi3Δdoa10Δ*, *opi3Δhrd1Δ*, and in *opi3Δusa1Δ* cells after blocking translation with cycloheximide. Proteins were separated by SDS-PAGE and detected by immunoblotting with antibodies against the HA tag and Tub1 as loading control.

The degradation of Sbh1 in *opi3Δhrd1Δ* and *opi3Δusa1Δ* was faster as compared to *opi3Δ*. One possibility is that the ERAD components from the unassembled *hrd1* complex can be recycled for the assembly of *doa10* complex, hence the possibility for the increased degradation of Sbh1. Another possibility is an increase in UPR activation

of cells under such conditions, resulting in increased ERAD activity which rapidly degrades Sbh1.

4.7. Biocomputational simulations suggest a Z directional movement of Sbh1 under LP

To further understand how Sbh1 is destabilised from the absence of PC, we collaborated with Dr. Haibin Su group in the Division of Materials Science to biocomputationally simulate Sbh1 embedded in a lipid bilayer with lipid compositions modelling those in WT and LP. As the structure of Sbh1 in *Saccharomyces cerevisiae* is currently still unknown, we modelled Sbh1 according to its homologue, Sec β in archaea. To ensure that Sbh1 transmembrane domain is conserved, 90 homologues of different species were aligned. The relative occurrence of amino acids at the respective positions with reference to the sequence of Sbh1 from *S. cerevisiae* was determined (Figure 4.6). Sbh1 transmembrane domain was found to be highly conserved among the different species. However, a limitation was that the modelled Sec β 's cytosolic domain is missing from the structure (1-44 residues).

Modelling of Sbh1 biocomputationally was performed in an all-atom molecular dynamics model, using the forcefield Gromos53a6. All the atoms in the molecules were represented and allowed for interaction in a fixed time period of 40 nanoseconds, providing a dynamic view of the trajectories of molecules in it. The forcefield Gromos53a6 is used in protein simulations and allow for the reproduction of free enthalpies in water, a strong interacting force present in biological membranes (Oostenbrink, Villa, Mark, & van Gunsteren, 2004). To mimic PC/PE in Δopi3 cells, pure 1,2-Bis(diphenylphosphino)ethane (DPPE) was done as a simplified representative of LP. A significant movement (0.6 Å) of Sbh1 in the Z direction and

only in pure DPPE bilayer member was energetically favourable (Figure 4.7). The movement in the Z directional plane depicts the unidirectional perpendicular movement of Sbh1 out of the membrane lipid bilayer towards the cytosol. No significant movement of Sbh1 in dipalmitoylphosphatidylcholine (DPPC) and the 'mix' lipid composition is found along the Z direction, with pure DPPC exhibiting a slight increase in stability as compared to the 'mix' composition. The movement of Sbh1 in pure DPPE along the X and Y direction, the directions along the lipid bilayer, is not significant (Figure S2). The results indicate that exposed hydrophobic residues normally embedded in the lipid bilayer can be exposed under lipid disequilibrium, marking Sbh1 for degradation.

Figure 4.6. Sequence conservation of Sbh1 transmembrane domain.

Sbh1 transmembrane domain of 90 different eukaryotes were aligned. The top three amino acids corresponding to each yeast Sbh1 position are shown.

Figure 4.7. Sbh1 exhibits significant movement along the Z direction in DPPE only lipid composition.

All-atom molecular dynamics (MD) model, Forcefield Gromos53a6 of Sec β in pure DPPC (dotted, 118 molecules), pure DPPE (coloured, 118 molecules) and a mixture of DPPE and DPPC of ratio 55 DPPC to 63 DPPE (outlined, 118 molecules). The mixture of DPPE and DPPC simplify WT conditions, while pure DPPE as *opi3Δ* conditions. DPPE, 1,2-Bis(diphenylphosphino)ethane; DPPC, dipalmitoylphosphatidylcholine.

The biocomputational simulation uses artificially modelled membrane lipid composition, as well as artificially modelled environmental water molecules. Sbh1 modelled in this simulation is also truncated and predicted according to its homologue, Sec β in archaea. Sbh1 can be purified to be used for *in vitro* modelling. This would allow further confirmatory experiments to be done using purified Sbh1 under different lipid composition extracted from microsomes to detect for similar movement.

4.8. A cytoplasmic stretch of Sbh1 is recognised and degraded by ERAD under lipid imbalance

To further elucidate how Sbh1 might be recognised for degradation, we looked into the conservation of the lysine residues on the cytoplasmic domain of Sbh1. We reported the frequency of occurrence of lysine residues at these positions (Figure 4.8). Lysine 41 and lysine 23 were found to be the most conserved, with 48.4% and 39.6%

occurrence, respectively. Lysine 23 was reported to be a likely site for ubiquitination together with lysine 31 (Swaney et al., 2013).

Figure 4.8. Sequence conservation of cytosolic lysine residues.

Sbh1 was aligned with 90 other homologues and the occurrence of the top three amino acids corresponding to the cytosolic lysine residues (shown in red) of Sbh1 are shown.

We mutated a key Sbh1 lysine residue that is near the ER membrane on the cytosolic site. The lysine at position 41 was mutated [Sbh1(K41A)] as its exposure to E3 ligase Doa10 might be prolonged in *opi3Δ* cells due to a decrease in membrane fluidity. Additionally, the shortening of the fatty acid chain of the ER (Figure 3.10) can result in an increase in exposure of the lysine residue. As expected, Sbh1(K41A) was significantly stabilised compared to Sbh1 in *opi3Δ* cells (Figure 4.9). In WT cells, no significant changes were observed in the stability of Sbh1(K41A) compared to Sbh1. This suggests that a degradation motif of TPs might become accessible to E3 ligases during LP leading to its premature degradation. It is also possible that the

transmembrane domain of Sbh1 might be misfolded under LP, leading to its premature degradation, where ubiquitylation of cytoplasmic lysine residues occur too (Briant, Koay, Otsuka, & Swanton, 2015)

Figure 4.9. Sbh1 is recognised and degraded by an exposed cytoplasmic stretch under LP.

The degradation of Sbh1-HA or Sbh1(K41A)-HA was analysed in WT and *opi3Δ* cells after blocking translation with cycloheximide. Proteins were separated by SDS-PAGE and detected by immunoblotting with antibodies against the HA tag and Tub1 as loading control.

4.9. Premature degradation of Sbh1 can result in the translocation defect of cells under LP

We have previously reported protein biogenesis defects of CPY and Gas1 in cells under LP without the intervention of the UPR (Thibault et al., 2012). Surprisingly, protein analysis revealed Sbh1 as the only protein candidate to have a lower abundance under LP in the co-translational and post-translational translocation complexes. Hence, we wanted to validate whether low abundance of Sbh1 can lead to the translocation defect of CPY. Translocation efficiency can be monitored *in vivo* by the shift in protein mobility on SDS-PAGE reflecting the addition of N-linked glycans in the ER. We confirmed that the absence of Sbh1 causes a delay in translocation from the increase in appearance of “pre” forms at the zero-minute time point in both *sbh1Δ* and *ire1Δsbh1Δ* mutants (Figure 4.10). This suggest that low abundance of Sbh1

under LP can result in the translocation defect seen in cells under LP. *ire1Δsbh1Δ* exhibits a similar translocation defect as *sbh1Δ*, indicating that the UPR might not be activated from the knocking out of *SBH1*.

Figure 4.10. A translocation defect occurs in *sbh1Δ* without the intervention of the UPR.

Cells were grown to early log phase in selective synthetic complete media before being pulse-labelled with L-[³⁵S]-methionine/cysteine for 5 min followed by a chase at the indicated times. Immunoprecipitated proteins using anti-CPY were resolved by SDS-PAGE and visualized by phosphorimager analysis. Positions of the non-translocated, ER, Golgi and mature forms of CPY were indicated by pre-CPY, p1, p2 and m, respectively.

We verified that *sbh1Δ* mutant did not activate the UPR significantly using a β -galactosidase reporter assay to detect for UPR activation (Figure 4.11). Under UPR activation, the upregulation of the other components of the post-translational translocation complex might be sufficient to overcome the delay in translocation, and no translocation deficiency was found in *opi3Δ* and *opi3Δsbh1Δ* mutants.

4.10. *SBH1* overexpression is not sufficient to fix translocation defect under lipid disequilibrium

The UPR is essential for cells under lipid disequilibrium, and LP mutants *CHO2* and *OPI3* exhibit synthetic lethality with UPR signalling gene *IRE1* (Costanzo et al., 2010). Hence, temperature sensitive alleles of *cho2-1* and *opi3-1* were generated in our laboratory. LP was found to result in a translocation defect without the intervention of the UPR, with a translocation defect observed in *cho2-1ireΔ* (Thibault et al., 2012). A

translocation defect was not detected in *opi3-1ire1Δ*. As the alleles generated exhibit varying degrees of loss of function of the alleles, the alleles in *opi3-1ire1Δ* generated might exhibit a milder degree of loss of function than the alleles generated in *cho2-1ire1Δ*. Pre-CPY was found to accumulate at the same ratio in the presence of Sbh1 under the control of *TDH3* promoter as compared to its endogenous promoter, signifying that overexpressing (OE) *SBH1* did not fix the post-translational translocation defect observed under LP (Figure 4.12 A). Similarly, the overexpression of Sbh1 fails to fix the post-translational translocation defect in Gas1, a glycosylphosphatidylinositol (GPI) anchor protein (Figure 4.13). Intriguingly, protein processing was seen to be expedited with OE *SBH1* for CPY, where the ratios of ER and Golgi apparatus forms are greatly reduced at the 15 and 30 min time point as compared to endogenous expression level. However, increased protein processing is not detected for Gas1 (Figure 4.13). We proposed an unreported role that Sbh1 is involved in protein processing for certain protein types. Overexpressing *SBH1* did not fix the co-translational translocation defect observed under LP similarly (Figure 4.12 B). The dissociation of Sbh1 with the Sec61 complex under LP (Figure 4.1 B) might have resulted in the translocation defect observed. The low abundance of Sbh1 under LP similarly can result in the translocation defect seen under LP. Taken together, Sbh1 becomes dissociated from the Sec61 complex under LP, causing it to be prematurely degraded by the Doa10 complex through ERAD, which can lead to translocational deficiencies of the cell (Figure 4.14).

Figure 4.11. *sbh1Δ* did not significantly upregulate the UPR.

Cells were grown to early log phase at 30°C in selective synthetic complete media. UPR induction was measured using a *UPRE-LacZ* reporter assay. Tm, tunicamycin.

Figure 4.12. Overexpression of *SBH1* in *cho2-1ire1Δ* did not fix the translocation defect observed but revealed a novel role in protein processing.

Cells were grown to early log phase in selective synthetic complete media before being pulse-labelled with L-[³⁵S]-methionine/cysteine for 5 min followed by a chase at the indicated times. Immunoprecipitated proteins using anti-CPY were resolved by SDS-PAGE and visualized by phosphorimager analysis. (A) Positions of the non-translocated, ER, Golgi and mature forms of CPY were indicated by pre-CPY, p1, p2 and m, respectively. (B) Positions of non-translocated and translocated forms of D_{HC}-α-F were indicated by pD_{HC}-α-F and gD_{HC}-α-F respectively.

Figure 4.13. Overexpression of *SBH1* in *cho2-1ire1*Δ did not fix the translocation defect in *Gas1*.

Cells were grown to early log phase in selective synthetic complete media before being pulse-labelled with L-[³⁵S]-methionine/cysteine for 5 min followed by a chase at the indicated times. Immunoprecipitated proteins using anti-*Gas1* were resolved by SDS-PAGE and visualized by phosphoimager analysis. (A) Positions of the non-translocated, ER and Golgi of *Gas1* were indicated by pre-*Gas1*, ER *Gas1*, and Golgi/PM *Gas1*, respectively.

4.11. Discussion

We have reported the changes in interaction of *Sbh1* under LP (Figure 4.1). The interactome of *Sbh1* greatly decreased under LP, including partners of the *Sec61* complex. Such changes in interactions can be adequately captured by the MYTH assay due to it being sensitive to transient interactions and the assay being performed *in vivo*.

Isolation of microsomes can result in changes in the properties of the biological membranes. As protein interactions have to be captured before isolation of microsomes, co-immunoprecipitation of Sbh1 with the usage of cross-linkers was performed. With the usage of cross-linker DTBP, we detected interaction of Sbh1 with Sec61 retained under LP (Figure 4.3). Cross-links might introduce a permanent relationship where there may not be one (Kluger & Alagic, 2004). In addition, the reagent itself may also induce new electrostatic or hydrophobic attraction, causing sites to react with each other that normally are not in proximity. It might be possible that Sbh1 interacted with Sec61 due to such new electrostatic or hydrophobic attraction introduced by the cross-linker DTBP. Taken together, the data supports the notion that the proteins conformation remains intact but Sbh1 interactions with other proteins got disrupted under LP.

We showed decreased membrane fluidity can result under PC depletion whereby Sbh1 becomes destabilised (Figure 3.12 and 3.13). Changes in membrane properties might have resulted in the change of interactome of Sbh1 under LP. Decreased fluidity of the membrane can result in increased lipid interactions between phospholipids in the membrane bilayer. TPs are regulated by the modulation of three competing forces; protein-lipid interaction, lipid-lipid interaction, and protein-protein interaction (Volmer & Ron, 2015). Increase in lipid-lipid interaction might have decreased the other opposing forces such as protein-protein interaction, resulting in the decrease in interaction of Sbh1 with the Sec61 complex. Under some circumstances, dissociated Sbh1 might be recognised for rapid degradation as seen with Sbh2 when dissociated from the Ssh1 complex (Habeck et al., 2015).

Additionally, the exposure of Sbh1 to the E3 ligase Doa10 may be prolonged under low membrane fluidity which can lead to its premature degradation. Lastly, the shortening

of the acyl chains of the phospholipids (Figure 3.10) together with the Z directional displacement of Sbh1 (Figure 4.7) might have exposed certain hydrophobic motif close to membrane bilayer, resulting in the protein being recognised and rapidly degraded by the ERAD complex. Further confirmatory experiments can be done using purified Sbh1 under WT and *opi3Δ* microsomal lipid composition to detect for similar movement in vitro.

Figure 4.14. Sbh1 dissociates from the Sec61 complex and is prematurely degraded under LP.

Conclusion of Sbh1 under LP. Under LP, Sbh1 becomes dissociated from the Sec61 complex and is rapidly recognised and degraded by the Doa10 complex. This results in lipid perturbed cells facing translocation deficiencies.

We have also reported novel interactions of Sbh1 that were picked out by the MYTH screen (Figure 4.1 A). We have picked out numerous interactors that were part of lipid metabolism; Alg3, Erg4, Erg24, Inp54, Nsg1, Scs2, Scs7 and Tsc13. It may be of interest to know if Sbh1 is involved in the regulation of lipid metabolism. Interesting interactors involved in the ER membrane morphology as was also found. Yop1 is a

reticulon-interacting protein involved in the generation of tubular ER morphology, and its physical interaction with Sbh1 have been verified (Miller et al., 2005). The interaction of Sbh1 with proteins involved in ER morphology might indicate a relationship between protein translocation and ER structure, and it is of interest to know whether they are dependent on each other.

Sbh1 was found to interact with both Ost4 and Kre2 in WT cells, but only with Kre2 in *opi3Δ* cells. Ost4 is a subunit of the evolutionarily conserved oligosaccharyl transferase complex (OST) complex which catalyses protein asparagine-linked glycosylation, and its physical interaction with Sbh1 have been verified (Chavan et al., 2005). Kre2 functions as a α 1,2-mannosyltransferase in the Golgi apparatus and is involved in protein mannosylation (Hausler, Ballou, Ballou, & Robbins, 1992). A protein processing delay occur when Sbh1 is in low abundance. The processing delay can be observed by tracking CPY in the different mutant strains. At the 15 min time point, *sbh1Δ* exhibited a large accumulation of p1 and p2 states of CPY, where most of the CPY in WT might be cleaved into its mature state (Figure 4.10). Accordingly, *opi3Δ* exhibited a similar trend where sbh1 levels are low under LP (Figure 3.2). The overexpression of Sbh1 is also able to fix the processing delay seen under *cho2-1ire1Δ* mutants (Figure 4.12), validating that Sbh1 plays a role in protein processing. This is interesting as Sbh1 remains interacting with Kre2 under LP, and this interaction can be responsible for the alleviation of the processing delay (Figure 4.1 B). However, this is not seen in Gas1, a GPI anchor protein, suggesting it might be processed independently of Sbh1 (Figure 4.13).

Several groups have reported that Sbh1 can play a role in protein processing. Physical interactions between Sbh1 and components of the exocyst complex and the OST complex have been reported (Chavan et al., 2005; J. H. Toikkanen, Miller, Soderlund,

Jantti, & Keranen, 2003). This suggest that Sbh1 can act as a mediator bringing the different complexes together, increasing the efficiency and reduce the duration of protein modifications and packaging. In eukaryotic cells, when the polypeptide is translocating into ER, the OST complex covalently transfers the N-glycan, Glc₃Man₉GlcNAc₂ (glucose₃-mannose₉-N-acetylglucosamine₂), to the Asn of the specific Asn-X-Ser/Thr motif (X any amino acid but not Proline) (Helenius & Aebi, 2004). As Sbh1 participate in both co-translational as well as post-translational translocation (Figure 1.7), its direct interaction with the OST complex can assist in both co-translational and post-translational manners. We reported the novel interaction of Sbh1 and Kre2 under WT as well as under LP. Protein mannosylation is required in various cellular processes (Timpel, Strahl-Bolsinger, Ziegelbauer, & Ernst, 1998) and is responsible for ER quality control by promoting the degradation of proteins which failed to fold after repeated attempts (Xu, Wang, Thibault, & Ng, 2013).

We would like to verify that the translocation defect seen under LP is caused by the premature degradation of Sbh1. We have generated a mutant of Sbh1, Sbh1(K41A), which was found to be significantly more stable under LP as compared to WT Sbh1 (Figure 4.9). It might be worth investigating if expression or the overexpression of Sbh1(K41A) is able to fix the translocation delay seen under LP (Figure 4.12). Besides protein translocation, we proposed that Sbh1 plays a dual role in protein processing which is revealed under LP model (Figure 4.12 A). The interaction of Sbh1 and Kre2 can be explored further to verify whether the alleviation of the processing delay from the OE of Sbh1 under LP is dependent on this interaction (Figure 4.1 B). Lastly, mass spectrometric analyses can be employed to investigate the change in protein modifications in WT and *sbh1*Δ cells.

Chapter 5. Characterization of Plasmodium phospholipid genes expressed in yeast

5.1. Introduction

Plasmodium falciparum is a unicellular protozoan parasite of humans that leads to malaria. Other species of the parasite include *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and the simian *Plasmodium knowlesi*. The parasites are transmitted through the female Anopheles mosquito, and are responsible for an estimated 438,000 deaths worldwide in 2015 (Cowman, Healer, Marapana, & Marsh, 2016; World Health Organization, 2015b). While this is a reduction from an estimated 554,000 deaths in 2010 as well as 738,000 deaths in 2005, malaria remains the seventh leading cause of deaths in low-income economies (World Health Organization, 2015a).

Malaria was traditionally treated by chloroquine or quinine, but with declining success (Bloland et al., 1993; Dondorp et al., 2005). The discovery of Artemisinin from *Artemisia annua* extract has sparked the development of a new class of antimalarial agents that is highly effective against the malaria parasites at their early stages of development. The treatment of Artemisinin has a tremendous impact on the mortality rates of malaria, and the 2015 Nobel Prize in Physiology or Medicine was jointly awarded to Youyou Tu for the discovery of Artemisinin (Nosten & White, 2007).

The onset of malaria can be summarized into four stages (Biamonte, Wanner, & Le Roch, 2013). Once the parasites from the mosquito invade the human blood stream, the parasites enter the liver and begin replicating there within 30 minutes. In the next stage after approximately 5-10 days, the parasites escaped from the liver cells and re-

enter the blood stream to invade red blood cells. They rapidly infect red blood cells and proliferate asexually. After several rounds of asexual reproduction, some parasites differentiate into male and female gametocytes. In the final stage, the ingestion of the gametocytes by mosquitoes will cause their fusion to form a zygote and develop into new parasites to infect other human hosts.

Artemisinin and its derivatives cause parasite death by indiscriminately binding to proteins in many of the parasites' key biochemical pathways. They are activated by heme, a component found in red blood protein haemoglobin, and cause lipid peroxidation by releasing free radicals (Meshnick, 2002). Being highly reactive and fast acting, Artemisinin and its derivatives eliminate the parasites quickly and make them highly effective against malaria.

Artemisinin-based combination therapies (ACTs) comprise of the usage of rapid acting Artemisinin with slow-clearing drugs such as artemether and lumefantrine as an effective combination to kill the parasites. This is done via targeting the malaria parasites at different stages in their life cycles (Biamonte et al., 2013). It is currently the first line of treatment against malaria and has been mostly effective (Eastman & Fidock, 2009; Mutabingwa, 2005). However, there are cases where malaria parasites became resistant to ACTs and such cases are rising in numbers (Ajayi & Ukwaja, 2013; Menard & Dondorp, 2017; Rosenthal, 2003; Satimai et al., 2012). While possibilities of developing triple combination therapies open up (Dipanjan, Shivaprakash, & Balaji, 2017), in depth understanding of the parasites' life cycle can bring out novel approaches in the elimination of the parasites.

One chemotherapeutic strategy to combat *Plasmodium falciparum* arises from the need of the parasites to increase phospholipid synthesis for membrane biogenesis and

cell division in the red blood cells. The phospholipid content of malaria-infected erythrocytes dramatically increases during maturation, with 85% of the newly phospholipids synthesised being PC and PE for growth and multiplication (Holz, 1977). Hence, the inhibition of phospholipid synthesis is an effective strategy for antimalaria therapeutics (Bobenchik et al., 2010; Pessi & Ben Mamoun, 2006). In addition, Artemisinin resistance is found associated with increased expression of UPR pathways, where UPR activation serves to mitigate protein damage caused by Artemisinin (Mok et al., 2015). We reported in Chapters 3 and 4 that LP compromises the UPR-mediated ER homeostasis by destabilising a subset of TPs which are part of the UPR programme. Thus, targeting phospholipid biosynthesis in combination with artemisinin might be an efficient strategy to overcome resistance by preventing effective UPR activation in *P. falciparum* (Ben Mamoun, Prigge, & Vial, 2010).

However, genes involved in the phospholipid pathways remain poorly characterised in *P. falciparum*, partially due to the adenine and thymine rich gene content in the parasite's genome (81%) (Gardner et al., 2002). As phospholipid pathways are highly conserved in eukaryotes (Carman & Henry, 1989), genetic complementation of *P. falciparum* genes in yeast have been used as a strategy to characterise them (Choi, Augagneur, Ben Mamoun, & Voelker, 2012; Choi et al., 2016).

A collaboration with Yaw Aniweh from Peter Preiser group in School of Biological Sciences was set up to characterise an unknown gene, *PF3D7_1143800* of strain 3D7 in *P. falciparum*. We termed this gene as *PfFMP*. The gene is conserved in *Plasmodium* species with an open reading frame, but the function of the protein it encodes has not been discovered. By performing a BLAST on a functional domain of the protein sequence, the gene in *Saccharomyces cerevisiae*, *FMP30*, came out as a top hit.

FMP30 encodes an inner mitochondria protein which plays a role in maintaining mitochondrial morphology as well as maintaining normal cardiolipin levels (Kuroda et al., 2011). Being involved in N-acylethanolamine metabolism, it might participate in protecting against oxidative stress damage (Merkel, Schmid, Paltauf, & Schmid, 2005). With Artemisinin causing parasite death by free radical damage, further understanding of N-acylethanolamine metabolism might aid in understanding and combating ARTs resistance strains of *plasmodium*. The knockout of the gene *FMP30* was reported to have minimal impact on the growth of the cells, which will not provide us with an observable phenotype to perform the genetic complementation assay with *PfFMP*. However, it was reported that the double mutant *psd1Δfmp30Δ* displayed a three-fold decreased growth rate as compared to WT (Figure 5.1 A). The growth of the *psd1Δfmp30Δ* is found to be impeded at 37°C, as well as under non-fermentable media YPAL (Figure 5.1 B). Under non-fermentable carbon source, yeast cannot metabolise lactate through anaerobic fermentation, hence requiring the mitochondria for aerobic respiration. As the growth defect is observable when compared to the control strains, the double mutant *psd1Δfmp30Δ* will be suitable for the expression of *PfFMP* to investigate for genetic complementation.

The double mutant *psd1Δfmp30Δ* was generated and the *PfFMP* was cloned into the pRS315 vector under the highly expressed *PGK1* promoter. To ensure that the plasmid is retained by the cells, synthetic media without leucine (SC-LEU) was used for Leucine selection of the vector.

Figure 5.1. *fmp30Δpsd1Δ* exhibits slow growth rates as compared to WT.

(A) Growth curves of *fmp30Δpsd1Δ* cells compared to WT, *psd1Δ*, and *fmp30Δ* in liquid YPAD medium at 30°C. The cultures were inoculated to an OD₆₀₀ of 0.05/ml from pre-cultures, and cell growth was monitored by measuring OD₆₀₀ at the indicated times. (B) Growth of *fmp30Δpsd1Δ* cells in different conditions. Cells were spotted onto a YPAD, YPAL, SCA or SCA containing 25 µg/ml ethidium bromide (EtBr) plate at fivefold serial dilutions starting with a density of 1.0 OD₆₀₀ unit/ml, and incubated at 30°C or 37°C for 3 days. (Adapted from Kuroda et al., 2011)

5.2. *PfFMP* complementation rescue growth defect of *psd1Δfmp30Δ*

We monitored the growth of *psd1Δfmp30Δ* expressing the *P. falciparum* gene *PfFMP*. Interestingly, the complementation of the homologue rescued the growth defect of *psd1Δfmp30Δ* to that of the single mutant *psd1Δ* (Figure 5.2). This provides strong evidence that the encoded protein of *PfFMP* has a similar function as Fmp30 in yeast. We noticed a growth defect in *psd1Δ* which was absent in the previous study (Kuroda et al., 2011). This can be explained from the different growth media, where we used synthetic media as compared to rich YPAD media, which is a complex yeast growth

media. Lipid precursors including ethanolamine are present in YPAD which are absent in synthetic media, and ethanolamine can be synthesised to PE via the Kennedy pathway (Figure 1.3). Hence *psd1Δ* cells grown in YPAD might have synthesised sufficient PE and exhibited no growth defect (Figure 5.1). the presence of *PSD2*, the homologue of *PSD1*, only accounts for 4-12% of total cellular phosphatidylserine decarboxylase activity, and might be insufficient alone to contribute to PE synthesis in synthetic media (Trotter & Voelker, 1995).

Figure 5.2. *PfFMP* rescues the growth defect of *fmp30Δpsd1Δ*.

Growth curves of *fmp30Δpsd1Δ* expressing *PfFMP* cells in SC-LEU medium at 30°C with WT, *psd1Δ*, *fmp30Δ*, *fmp30Δ* expressing *PfFMP*, and *fmp30Δpsd1Δ* strains. The cultures were inoculated to an OD₆₀₀ of 0.05/ml from pre-cultures, and cell growth was monitored by measuring OD₆₀₀ at the indicated times.

Spotting assays on SC-LEU plates were performed with the strains at 30°C or 37°C for 3 days. Similar to the growth assay, a growth defect in *psd1Δ* mutant is observed (Figure 5.3). The complementation of *PfFMP* was found to rescue the growth defect of *fmp30Δpsd1Δ* at 30°C, but the rescue was abolished at 37°C. *psd1Δ* strains exhibit a severe growth defect under 37°C as reported in previous study (Storey et al., 2001).

Catalysing the decarboxylation of phosphatidylserine (PS) to produce PE, mitochondrial *PSD1* accounts for approximately 90% of total cellular phosphatidylserine decarboxylase activity, while *PSD2* catalyses the remaining 10% in the Golgi apparatus. The growth defect is attributed to the presence of low levels of PE, and supplementing the growth medium with ethanolamine is able to alleviate this defect (Birner, Burgermeister, Schneiter, & Daum, 2001).

Figure 5.3. *PfFMP* alleviates the growth defect under 30°C but not 37°C.

Growth of *fmp30*Δ*psd1*Δ + *PfFMP* in SC-LEU medium with WT, *psd1*Δ, *fmp30*Δ, *fmp30*Δ + *PfFMP*, and *fmp30*Δ*psd1*Δ strains. Cells were spotted on SC-LEU plate at fivefold serial dilutions starting with a density of 0.2 OD₆₀₀ unit/ml and incubated at 30°C or 37°C for 3 days.

5.3. The yeast *PSD1* has a homologue *PfPSD* in *P. falciparum*

Interestingly, a homologue of *PSD1* in *S. cerevisiae* was found in *P. falciparum* denominated as PF3D7_0927900 from performing a BLAST. We termed this gene as *PfPSD*. *PfPSD* encodes the homologue of *Psd1* in yeast, which serve as a phosphatidylserine decarboxylase converting phosphatidylserine to PE. The gene was uncharacterised when we started but have later been characterised. (Choi et al.,

2016). The disruption of this gene can cause LP through the blocking of the synthesis of PE. *PfPSD* is also crucial for the development, differentiation and pathogenesis of malaria, and hence might be a suitable target for the development of antimicrobials. Hence, we proceeded to characterise this gene as well.

To characterise *PfFMP* as well as *PfPSD* in *P. falciparum*, we synthesised the codon optimized Open Reading Frame (ORF) of *PfFMP* and *PfPSD* with the yeast endogenous promoters of *FMP30* and *PSD1* respectively. Under their own endogenous promoters, the expression of the *P. falciparum* will be similar to the yeast counterpart. The mitochondria localizing signal (MLS) of the yeast genes were added respectively to the N-terminus of the *PfFMP* and *PfPSD*. The MLSs were added to ensure that the *P. falciparum* genes are localised similarly to their yeast counterparts. In addition, a 3X HA tag was added to the C-terminus of the constructs to detect for their expression.

However, the MLS of *PfFMP* is truncated and the 3X HA tag is removed from the construct. The 39 amino acids MLS is truncated to 13 amino acids to disable its function. We also introduced a stop codon before the 3X HA tag of *PfFMP* at the C-terminus via site directed mutagenesis. These changes were performed as preliminary results revealed that the unmodified *PfFMP* exacerbated the growth defect of the double mutant *fmp30Δpsd1Δ*. The MLS as well as the 3X HA tag might have disrupted the protein conformation of *PfFMP*, resulting in the aggravating phenotype. Hence, *PfFMP* and *PfPSD* are constructed with these schematics to investigate for genetic complementation in yeast (Figure 5.4).

Figure 5.4. *PfFMP* and *PfPSD* were cloned with their yeast endogenous promoters and MLS.

Schematic design of the construct *PfFMP* and *PfPSD*. 500 bp upstream promoter is constructed together with the MLS of *FMP30* (truncated) and *PSD1* (derived from UniProt) and the ORF of *PfFMP* and *PfPSD* codon optimized to *S. cerevisiae*. A 3X HA tag is present in *PfPSD* but not *PfFMP*.

The expression of *PfPSD* was verified by immunoblotting through detection with HA antibodies (Figure 5.5). The majority of *PfPSD* was in the form of *PfPSD* proenzyme, indicating that newly synthesised *PfPSD* is not efficiently processed through an auto-endoproteolytic cleavage mechanism, but a substantial *PfPSD* mature β subunit band was still detected (Choi, Duraisingh, Marti, Ben Mamoun, & Voelker, 2015). However, we failed to verify the expression of *PfFMP* (*PfFMP* with 3 X HA tag previously constructed) by immunoblotting at 107 kDa. This might be due to the low expression profile under the yeast endogenous promoter *FMP30*, where around 200 proteins/cell is reported (Ghaemmaghami et al., 2003; Kulak, Pichler, Paron, Nagaraj, & Mann, 2014). Thus, we proceeded with complementation of the *P. falciparum* genes in yeast mutants.

5.6. *PfFMP* and *PfPSD* are both able to rescue the growth defect seen in *fmp30Δpsd1Δ*

We expressed *PfFMP* and *PfPSD* in the various yeast mutants and investigated their growth rates (Figure 5.6). The expression of *PfPSD* was able to rescue the growth defect in both *psd1Δ* as well as *fmp30Δpsd1Δ* mutants. Expressing *PfFMP* alleviated the growth defect in *fmp30Δpsd1Δ* substantially, while the expression of both *PfFMP* and *PfPSD* is able to significantly alleviate the growth defect of *fmp30Δpsd1Δ*.

The yeast strains were spotted at 30°C and 37°C in SC-LEU-URA plates. Similarly, the expression of both *PfFMP* and *PfPSD* is identical to the growth assay at 30°C, and is able to complement their respective strains accordingly (Figure 5.7). Under 37°C, *PfPSD* is able to alleviate the growth defect of *psd1Δ*, and both *PfFMP* and *PfPSD* is able to alleviate the growth defect of *fmp30Δpsd1Δ* mildly.

Figure 5.5. *PfPSD* expression was verified by western blotting.

PfPSD proenzyme and PfPSD mature β subunit were detected by western blotting using HA antibodies at 49 kDa and 43 kD in PfPSD expressing strains. PfFMP was not detected using HA antibodies at 107 kDa in PfFMP expressing strains. *unspecific band.

5.7. The expression of both *PfFMP* and *PfPSD* is able to restore depleted cardiolipin seen in *fmp30Δpsd1Δ*

It has been previously reported that Fmp30 plays a role in maintaining normal cardiolipin (CL) levels (Kuroda et al., 2011). A slight decrease in CL level was detected in *fmp30Δ*, but a 20-fold decrease in CL was detected in *fmp30Δpsd1Δ* mutant. We investigated whether the complementation of *PfFMP* and *PfPSD* in *fmp30Δpsd1Δ* was able to complement the decrease in CL. We isolated crude mitochondria of yeast and performed one dimensional thin layer chromatography to separate cardiolipins, phosphatidylglycerol (PG) and phosphatidic acid. We detected a restoration of CL levels in the complemented strain, as compared to the almost absent CL detected in *fmp30Δpsd1Δ* mutant (Figure 5.8). A drastic decrease in PG was observed in

fmp30Δpsd1Δ but became restored with genetic complementation. CL is synthesised from PG with cytidine diphosphate, hence the reduction in PG might have affected CL synthesis (Schlame, Brody, & Hostetler, 1993). *psd1Δ* has little to no impact on PG (Bottinger et al., 2012), and hence the restoration of PG levels might be attributed to the complementation of the *PfFMP* gene.

Figure 5.6. *PfFMP* and *PfPSD* are able to rescue the growth defect in *fmp30Δpsd1Δ*.

(A) Growth curves of *PfFMP* and *PfPSD* complemented strains in SC-LEU-URA medium at 30°C. The cultures were inoculated to an OD₆₀₀ of 0.05 from pre-cultures, and cell growth was monitored by measuring OD₆₀₀ at the indicated times. Error bars show the SE of three technical replicates. (B) The exponential phase of the growth curve is calculated. * $P < 0.05$, ** $P < 0.001$, $P > 0.05$ denoted as ns; non-significant, Student's t test.

5.8. Discussion

Genetic complementation in yeast has been used extensively with success in characterizing unknown genes of various organisms, including *Arabidopsis thaliana*, *Drosophila melanogaster*, *Plasmodium falciparum* and *Homo sapiens* (Choi et al., 2016; Henikoff, Tatchell, Hall, & Nasmyth, 1981; Riou, Tourte, Lacroute, & Karst, 1994; Zhou & Gitschier, 1997). The conservation of lipid metabolic pathways in eukaryotes

as well as the importance of lipid metabolism in proliferation of the parasites makes this strategy all the more promising where such pathways identified can be potential targets for future drug discovery (Carman & Henry, 1989; Gulati et al., 2015).

Figure 5.7. *PfFMP* and *PfPSD* are able to alleviate the growth deficiency seen in *fmp30Δpsd1Δ*.

PfFMP and *PfPSD* complemented strains were spotting on SC-LEU-URA medium at 30°C and 37°C. Cells were spotted at fivefold serial dilutions starting with a density of 0.2 OD₆₀₀ unit/ml and incubated at 30°C or 37°C for 3 days.

Fmp30 has been proposed to be involved in N-acylethanolamine (NAE) metabolism and shared homology with the human NAPE-specific phospholipase D (Merkel, Schmid, Paltauf, & Schmid, 2005). NAE has been proposed to be involved in signalling or have cytoprotective effects (Fowler & Jacobsson, 2002; Hillard & Jarrahan, 2000). The genetic complementation of *PfFMP* in yeast was found able to alleviate the grow found in *fmp30Δpsd1Δ* strain (Figures 5.6 and 5.7). *PfFMP* together with *PfPSD* was able to restore CL as well as PG levels in *fmp30Δpsd1Δ*. These provide evidences that *PfFMP* can complement *FMP30*. However, we are unable to rule out any indirect effect

that *PfFMP* and *PfPSD* that might have which restored CL and PG levels in *fmp30Δpsd1Δ*. Hence, *PfFMP* might be the homologue of Fmp30 in yeast and have similar functions. Further studies can be carried out to investigate the cytoprotective effects of *PfFMP* in malaria to further characterise the gene. As Artemisinin and its derivatives kill the parasites through free radicals, *PfFMP* might play important roles for the parasites to be resistance to ACTs, and hence can be inhibited to induce sensitivity of the parasites to ACTs.

Figure 5.8. *PfFMP* and *PfPSD* complementation restore CL as well as PG levels in *fmp30Δpsd1Δ*.

One dimensional thin Layer chromatography of cardiolipins, phosphatidylglycerol and phosphatidic acid. Crude mitochondria fraction was isolated from yeast strains by differential centrifugation before lipid extraction and lipids were separated using the solvent mixture chloroform:hexane:methanol:acetic acid on a 20 cm x 20 cm Silica gel 60G F₂₅₄ glass plate. CL, cardiolipin; PG, phosphatidylglycerol; O, origin.

PfPSD encodes the homologue of Psd1 in yeast, which serve as a phosphatidylserine decarboxylase converting phosphatidylserine to PE (Choi et al., 2016). *PfPSD* has been found to rescue the growth defect of *psd1Δ* and *fmp30Δpsd1Δ* (Figures 5.6, 5.7). The need for the increase in phospholipid synthesis during propagation of the parasites

for membrane biogenesis and cell division in the red blood cells makes this inhibition an effective strategy for antimalaria therapeutics (Bobenchik et al., 2010; Pessi & Ben Mamoun, 2006). Screening of potential anti-malarial drugs can be performed to identify drugs that can successfully inhibit the enzyme, while exhibiting low toxicity towards mammalian cells. The success in eliminating malaria infection of such drugs could then be further evaluated in mice.

Artemisinin resistance is also found associated with increased UPR expression, where the UPR activation serves to mitigate protein damage (Mok et al., 2015). The perturbation of PC/PE has been found to result in the compromise of the UPR (Chapters 3 and 4). Psd1 catalyses 90% of the decarboxylation of total cellular phosphatidylserine to produce PE (Storey et al., 2001). Hence, the disruption of the gene Psd1 would result in drastic reductions of PE synthesis. Elevated PC/PE levels which have been found to result in chronic ER stress (Fu et al., 2011) might result from decreased PC synthesis. Hence, inhibiting PE synthesis can contribute to the compromise of the UPR. As the UPR is a powerful system to mitigate protein damage, introducing LP might be an effective solution for killing parasites resistance to ACTs. Further works that investigate the effects of LP inducing drugs on ACTs resistance parasites can further confirm and reveal the effectiveness of such a line of treatment against the parasites.

Chapter 6. Conclusion and Future Directions

The strong association between lipid perturbation and various metabolic diseases such as obesity and non-alcoholic fatty liver disease (NAFLD) in human populations is evident of the importance in understanding lipid homeostasis and the consequences of lipid perturbation. NAFLD is now the most common cause of chronic liver enzyme elevations and cryptogenic cirrhosis, as a result of the increased prevalence of obesity. The same trend is seen in Singapore, where the increasingly pervasiveness of NAFLD seek public awareness as well as novel therapeutics (Khaw et al., 2016).

Chronic ER stress has been linked extensively to NALFD, as well as a whole assortment of metabolic diseases such as T2D, cardiovascular disease (CVDs), and cerebrovascular diseases (Gupta et al., 2010; McAlpine & Werstuck, 2013; Ozcan et al., 2004; Zhang, Xu, Yu, Chen, & Li, 2014). The UPR is solely responsible for the activation of multiple intracellular signalling pathways designed to restore the ER (and so cellular) homeostasis (Walter & Ron, 2011). As the ER is involved in numerous cellular functions and exposed to various conditions, the UPR provides a signalling framework into which other pathways are collectively integrated (Rutkowski & Hegde, 2010). Under chronic ER stress, the prolonged activation of the UPR can trigger apoptosis implicated in various diseases (Tabas & Ron, 2011).

In Chapter 3 and 4, we explored the consequences of prolonged LP, and showed that certain UPR upregulated TPs became destabilised under LP. Being involved in various pathways to mediate ER homeostasis, their premature degradation might prevent the UPR in restoring ER homeostasis. The failure to restore ER homeostasis can result in chronic ER stress where the UPR is activated continuously. As prolonged LP is found in various diseased models, the premature degradation of TPs might contribute to the

development of such diseased states (Arendt et al., 2013; Fu et al., 2011; Z. Wang et al., 2011).

The premature degradation of TPs under LP can be further verified in higher organisms such as mouse models or in diseased samples such as hepatocytes acquired from NAFLD patients. The translation of this work to its relevance in diseases can have a great impact on the understanding of how such metabolic diseases develop under prolonged LP. Phospholipid composition is highly conserved in the ER (Zinser et al., 1993), and the effects it exerts on TPs might be uniform from yeast to humans. In addition, UPR upregulated TPs can be screened in diseased models to check for their stability. The consequences for the premature degradation of such TPs can also be further investigated in how they contribute to the onset and progression of the disease.

The mechanism of how TPs are recognised and degraded rapidly can be further elucidated. In Chapter 3, we established that changes in the membrane properties such as the stiffening of the membrane might affect the stability of certain TPs. Establishing the mechanism for the destabilisation of TPs might open novel avenues and approaches in the alleviation of chronic ER stress from prolonged LP. In Chapter 4, we elucidated the mechanism in which Sbh1 became recognised and ubiquitinated for degradation under LP. TPs that are prematurely degraded by LP can be similarly recognised and degraded like Sbh1, and a common degradation machinery under LP can be explored. The underlying recognition and degradation might be common and shared amongst the TPs that are destabilised under LP. Understanding how certain TPs are more susceptible to LP can also allow further modelling and prediction of other TPs which putatively be likely candidates for destabilisation under other perturbed conditions.

A novel role of Sbh1 in protein processing has been unexpectedly uncovered in the course of our study mentioned in Chapter 4. Under LP, participation of Sbh1 in protein processing is seen when the overexpression of Sbh1 expedited CPY in reaching its mature form (Figure 4.12), while the loss of Sbh1 causes a delay in protein processing (Figure 4.10). The mechanism of Sbh1's involvement in protein processing can be further investigated. Sbh1 is found interacting with a number of proteins involved in protein modification (Figure 4.1), as well as with components of the exocyst complex (J. H. Toikkanen et al., 2003), suggesting that Sbh1 act as a mediator protein between the Sec61 translocon, OST complex, as well as the exocyst complex, expediting protein secretion. Indeed, the overexpression of Sbh1 has been found to increase secretion of proteins in a study (Toikkanen, Sundqvist, & Keranen, 2004). By exploring the functional roles of Sbh1 in the secretory pathway, its expression as well as its interacting partners can be manipulated to increase protein yield in protein synthesis platforms.

In Chapter 5, We characterised few *Plasmodium falciparum* genes involved in the regulation of phospholipids. Besides understanding the involvement of lipid perturbation in the development of metabolic diseases, knowledge of phospholipid regulation can be applied and translated into therapeutics against pathogens such as malaria. Coincidentally, the conclusion derived from Chapter 3 and 4 that LP compromises UPR-mediated ER homeostasis increase the promise of exploiting LP as therapeutics against certain pathogens. *P. falciparum* resistance to artemisinin-based combination therapies (ACTs) is associated to ER stress where the UPR mitigates artemisinin-induced protein damage (Mok et al., 2015). As the UPR is a powerful stress response typically conserved in most organisms (Hollien, 2013), UPR upregulation has been identified in other pathogens to resist the host innate immune system or drug treatments (Amano et al., 2003; Cheon et al., 2011; Richie et al., 2009; Smith, 2014). Similarly, it might be applied to diseases such as cancer where UPR

activation promotes cell survival and not apoptosis (Bi et al., 2005; Wu et al., 2014). In addition, increased phospholipid synthesis is usually coupled with rapid cell division for cell membrane synthesis (Bobenchik et al., 2010; DeBerardinis, Lum, Hatzivassiliou, & Thompson, 2008; Joseleau-Petit, Kepes, Peutat, D'Ari, & Kepes, 1987). Hence, the induction of LP can have a two-fold effect in such circumstances.

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Appendix

Table S1. Protein candidates that have decreased protein abundance despite being upregulated transcriptionally. Adapted from (Thibault et al., 2012).

Gene	Function	Localisation	Number of transmembrane domains
<i>ATE1</i>	Arginyl-tRNA-protein transferase; catalyzes post-translational conjugation of arginine to the amino termini of acceptor proteins	Cytoplasm	0
<i>ATG2</i>	Peripheral membrane protein required for autophagic vesicle formation	Vesicles	Peripheral membrane
<i>BMH2</i>	involved in regulation of many processes including exocytosis, vesicle transport, Ras/MAPK signalling, and rapamycin-sensitive signalling	Cytosol/Nucleus	0
<i>CCC2</i>	Cu(+2)-transporting P-type ATPase; required for export of copper from the cytosol into an extracytosolic compartment	Golgi membrane	8
<i>COX14</i>	Mitochondrial cytochrome c oxidase (complex IV) assembly factor; also involved in translational regulation of Cox1p and prevention of Cox1p aggregation before assembly	Mito membrane	1
<i>COY1</i>	Golgi membrane protein with similarity to mammalian CASP; genetic interactions with GOS1 (encoding a Golgi snare protein) suggest a role in Golgi function	Golgi membrane	1
<i>CPA1</i>	Small subunit of carbamoyl phosphate synthetase; carbamoyl phosphate synthetase catalyzes a step in the synthesis of citrulline, an arginine precursor	Cytosol	0
<i>CRN1</i>	Coronin; cortical actin cytoskeletal component that associates with the Arp2p/Arp3p complex to regulate its activity; plays a role in regulation of actin patch assembly	Cytoplasm	0

Gene	Function	Localisation	Number of transmembrane domains
<i>CRZ1</i>	Transcription factor, activates transcription of stress response genes; nuclear localisation is positively regulated by calcineurin-mediated dephosphorylation	Cytosol/Nucleus	0
<i>CTR1</i>	High-affinity copper transporter of plasma membrane; mediates nearly all copper uptake under low copper conditions; transcriptionally induced at low copper levels and degraded at high copper levels	PM	2
<i>CUE1</i>	Ubiquitin-binding protein; endoplasmic reticulum membrane protein that recruits the ubiquitin-conjugating enzyme Ubc7p to the ER where it functions in protein degradation	ER membrane	1
<i>EMC4</i>	Member of conserved ER transmembrane complex; required for efficient folding of proteins in the ER; null mutant displays induction of the unfolded protein response	ER membrane	2
<i>ENT2</i>	Epsin-like protein required for endocytosis and actin patch assembly; functionally redundant with Ent1p; contains clathrin-binding motif at C-terminus	PM	Peripheral membrane
<i>ERP5</i>	Protein with similarity to Emp24p and Erv25p; member of the p24 family involved in ER to Golgi transport. Involved in vesicular protein trafficking	ER membrane	1
<i>FET4</i>	Low-affinity Fe(II) transporter of the plasma membrane	PM	7
<i>FRQ1</i>	N-myristoylated calcium-binding protein; may have a role in intracellular signalling through its regulation of the phosphatidylinositol 4-kinase Pik1p; member of the recoverin/frequenin branch of the EF-hand superfamily	Bud membrane/PM ¹	1
<i>FUN14</i>	Mitochondrial protein of unknown function	Mito membrane	3

Gene	Function	Localisation	Number of transmembrane domains
<i>FZO1</i>	Mitofusin; integral membrane protein involved in mitochondrial outer membrane tethering and fusion; role in mitochondrial genome maintenance	Mito outer membrane	2
<i>GAP1</i>	General amino acid permease; Gap1p senses the presence of amino acid substrates to regulate localisation to the plasma membrane when needed; essential for invasive growth	PM	12
<i>GCD10</i>	Subunit of tRNA (1-methyladenosine) methyltransferase with Gcd14p; required for the modification of the adenine at position 58 in tRNAs, especially tRNA ⁱ -Met	Nucleus	0
<i>GIP3</i>	Cytoplasmic protein that regulates protein phosphatase 1 Glc7p; overexpression relocalises Glc7p from the nucleus and prevents chromosome segregation; may interact with ribosomes	Cytosol/ER	0
<i>GLN3</i>	Transcriptional activator of genes regulated by nitrogen catabolite repression; localisation and activity regulated by quality of nitrogen source and Ure2p	Nucleus	0
<i>HNT1</i>	Adenosine 5'-monophosphoramidase; interacts physically and genetically with Kin28p, a CDK and TFI κ subunit, and genetically with CAK1; member of histidine triad (HIT) superfamily of nucleotide-binding proteins	Nucleus ¹	0
<i>HOL1</i>	Putative transporter in the major facilitator superfamily; member of the 12-spanner drug:H(+) antiporter DHA1 family	Bud membrane/	11
<i>HSH49</i>	U2-snRNP associated splicing factor; similar to the mammalian splicing factor SAP49; proposed to function as a U2-snRNP assembly factor along with Hsh155p and binding partner Cus1p	Nucleus	0

Gene	Function	Localisation	Number of transmembrane domains
<i>ITR1</i>	Myo-inositol transporter; member of the sugar transporter superfamily; expression is repressed by inositol and choline via Opi1p and derepressed via Ino2p and Ino4p	ER membrane	12
<i>LDB7</i>	Component of the RSC chromatin remodeling complex; interacts with Rsc3p, Rsc30p, Npl6p, and Htl1p to form a module important for a broad range of RSC functions	Nucleus	0
<i>LEO1</i>	Component of the Paf1 complex; which associates with RNA polymerase II and is involved in histone methylation; plays a role in regulating Ty1 transposition	Nucleoplasm	0
<i>MAD2</i>	Component of the spindle-assembly checkpoint complex; delays onset of anaphase in cells with defects in mitotic spindle assembly; forms a complex with Mad1p	Nucleus	0
<i>MCA1</i>	Ca ²⁺ -dependent cysteine protease; may cleave specific substrates during the stress response; regulates apoptosis upon H ₂ O ₂ treatment; required for clearance of insoluble protein aggregates during normal growth	Cytosol/Nucleus	0
<i>MRS5</i>	Essential protein of the inner mitochondrial membrane; peripherally localised; component of the TIM22 complex, which is a twin-pore translocase that mediates insertion of numerous multispinning inner membrane proteins	Mito inner membrane	Peripheral membrane
<i>MSC3</i>	Protein of unknown function; green fluorescent protein (GFP)-fusion protein localises to the cell periphery; msc3 mutants are defective in directing meiotic recombination events to homologous chromatids; potential Cdc28p substrate	Cell membrane	Peripheral membrane

Gene	Function	Localisation	Number of transmembrane domains
<i>MSN4</i>	Stress-responsive transcriptional activator; activated in stochastic pulses of nuclear localisation in response to various stress conditions; binds DNA at stress response elements of responsive genes, inducing gene expression	Cytosol/Nucleus	0
<i>NBP2</i>	Protein involved in the HOG (high osmolarity glycerol) pathway; negatively regulates Hog1p by recruitment of phosphatase Ptc1p the Pbs2p-Hog1p complex	Cytosol	0
<i>NNF2</i>	Protein that exhibits physical and genetic interactions with Rpb8p; Rpb8p is a subunit of RNA polymerases I, II, and III; computational analysis of large-scale protein-protein interaction data suggests a role in chromosome segregation	Cytosol	0
<i>NSG2</i>	Protein involved in regulation of sterol biosynthesis; specifically stabilises Hmg2p, one of two HMG-CoA isoenzymes that catalyze the rate-limiting step in sterol biosynthesis	ER membrane	4
<i>NSP1</i>	FG-nucleoporin component of central core of the nuclear pore complex; also part of the nuclear pore complex (NPC) nuclear basket; contributes directly to nucleocytoplasmic transport and maintenance of the NPC permeability barrier	Nucleus	Peripheral membrane
<i>PBP4</i>	Pbp1p binding protein; interacts strongly with Pab1p-binding protein 1 (Pbp1p) in the yeast two-hybrid system; also interacts with Lsm12p in a copurification assay	Cytosol/Nucleus	0
<i>PGA2</i>	Essential protein required for maturation of Gas1p and Pho8p; involved in protein trafficking; GFP-fusion protein localises to the ER and YFP-fusion protein to the nuclear envelope-ER network	ER membrane	1
<i>PHM8</i>	Lysophosphatidic acid (LPA) phosphatase, nucleotidase; principle and physiological nucleotidase working on GMP, UMP and CMP;	Cytosol/Nucleus	0

Gene	Function	Localisation	Number of transmembrane domains
<i>PMT2</i>	Protein O-mannosyltransferase of the ER membrane; transfers mannose residues from dolichyl phosphate-D-mannose to protein serine/threonine residues; involved in ER quality control	ER membrane	9
<i>POB3</i>	Subunit of the heterodimeric FACT complex (Spt16p-Pob3p); FACT associates with chromatin via interaction with Nhp6Ap and Nhp6Bp, and reorganizes nucleosomes to facilitate access to DNA by RNA and DNA polymerases	Nucleus	0
<i>PRM5</i>	Pheromone-regulated protein, predicted to have 1 transmembrane segment; induced during cell integrity signalling	PM	1
<i>PRP46</i>	Member of the NineTeen Complex (NTC); this complex contains Prp19p and stabilises U6 snRNA in catalytic forms of the spliceosome containing U2, U5, and U6 snRNAs	Cytosol	0
<i>RPB4</i>	RNA polymerase II subunit B32; forms dissociable heterodimer with Rpb7p; Rpb4/7 dissociates from RNAPII as Ser2 CTD phosphorylation increases; Rpb4/7 regulates cellular lifespan via mRNA decay process	Cytosol/Nucleus	0
<i>RTT102</i>	Component of both the SWI/SNF and RSC chromatin remodeling complexes; suggested role in chromosome maintenance; possible weak regulator of Ty1 transposition	Nucleus	0
<i>SBH1</i>	Beta subunit of Sec61p ER translocation complex (Sec61p-Sss1p-Sbh1p); involved in protein translocation into the endoplasmic reticulum; interacts with the exocyst complex and also with Rtn1p	ER membrane	1
<i>SCD6</i>	Repressor of translation initiation; binds eIF4G through its RGG domain and inhibits recruitment of the preinitiation complex; also contains an Lsm domain; may have a role in RNA processing	Cytosol	-

Gene	Function	Localisation	Number of transmembrane domains
<i>SCS7</i>	Sphingolipid alpha-hydroxylase; functions in the alpha-hydroxylation of sphingolipid-associated very long chain fatty acids	ER membrane	4
<i>SPI1</i>	GPI-anchored cell wall protein involved in weak acid resistance; basal expression requires Msn2p/Msn4p; expression is induced under conditions of stress and during the diauxic shift	Cell Wall	GPI Anchor
<i>TAF11</i>	TFIID subunit (40 kDa); involved in RNA polymerase II transcription initiation	Nucleus	0
<i>TDH2</i>	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), isozyme 2; involved in glycolysis and gluconeogenesis; tetramer that catalyzes reaction of glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate	Cytoplasm	0
<i>TIM18</i>	Component of the mitochondrial TIM22 complex; involved in insertion of polytopic proteins into the inner membrane; may mediate assembly or stability of the complex	Mito outer membrane	3
<i>TRX2</i>	Cytoplasmic thioredoxin isoenzyme; part of thioredoxin system which protects cells against oxidative and reductive stress; required for ER-Golgi transport and vacuole inheritance	GA/Nucleus/Cytosol	Peripheral membrane
<i>URA4</i>	Dihydroorotase; catalyzes the third enzymatic step in the de novo biosynthesis of pyrimidines, converting carbamoyl-L-aspartate into dihydroorotate	Cytosol/Nucleus	0
<i>VNX1</i>	Calcium/H ⁺ antiporter localised to the endoplasmic reticulum membrane; member of the calcium exchanger (CAX) family; potential Cdc28p substrate	ER membrane	13
<i>VT11</i>	Protein involved in cis-Golgi membrane traffic; v-SNARE that interacts with two t-SNARES, Sed5p and Pep12p; required for multiple vacuolar sorting pathways; human homologue VT11A can complement yeast null mutant	Golgi membrane	1

Gene	Function	Localisation	Number of transmembrane domains
<i>YAP1</i>	Basic leucine zipper (bZIP) transcription factor; required for oxidative stress tolerance; activated by H ₂ O ₂ through the multistep formation of disulfide bonds and transit from the cytoplasm to the nucleus	Cytosol/Nucleus	0
<i>YDL173 W</i>	Putative protein of unknown function; hyperphosphorylated upon rapamycin treatment in a Tap42p-dependent manner	Cytosol	0
<i>YDR026 C</i>	RNA polymerase I termination factor; binds to rDNA terminator element, required for efficient Pol I termination; required for rDNA silencing at NTS1	Nucleus	0
<i>YET3</i>	Protein of unknown function; YET3 null mutant decreases the level of secreted invertase; homologue of human BAP31 protein	ER membrane	3
<i>YGR130 C</i>	Component of the eisosome with unknown function; GFP-fusion protein localises to the cytoplasm; specifically phosphorylated in vitro by mammalian diphosphoinositol pentakisphosphate (IP ₇)	Cytosol	0
<i>YJU2</i>	Essential protein required for pre-mRNA splicing; associates transiently with the spliceosomal NTC ("nineteen complex") and acts after Prp2p to promote the first catalytic reaction of splicing	Nucleus	0
<i>YNL157 W</i>	Protein required for initiation of G0 programme; prevents degradation of nutrient-regulated mRNAs via the 5'-3' mRNA decay pathway; phosphorylated by Rim15p	Cytosol/Nucleus	0
<i>YNL217 W</i>	Putative protein of unknown function; weak sequence similarity to bis (5'-nucleotidyl)-tetraphosphatases; (GFP)-fusion protein localises to the vacuole	Vacuolar	0
<i>ZDS2</i>	Protein with a role in regulating Swe1p-dependent polarized growth; involved in maintenance of Cdc55p in the cytoplasm where it promotes mitotic entry	Cytosol	0

*Gray background are are ER transmembrane proteins, ¹Putative

Figure S1. DNA microarray analysis of *CUE1*, *EMC4*, *NSG2* and *SBH1*.

CUE1, *EMC4*, *NSG2* and *SBH1* were upregulated in *opi3Δ* compared to WT. Adapted from (Thibault et al., 2012)

Figure S2. Sbh1 exhibit no significant movement along the X and Y directions in DPPE only lipid composition.

All-atom molecular dynamics (MD) model, Forcefield Gromos53a6 of Sec β in pure DPPC (dotted, 118 molecules), pure DPPE (colored, 118 molecules) and a mixture of DPPE and DPPC of ratio 55 DPPC to 63 DPPE (outlined, 118 molecules). The mixture of DPPE and DPPC simplify WT conditions, while pure DPPE as *opi3 Δ* conditions. DPPE, 1,2-Bis(diphenylphosphino)ethane; DPPC, dipalmitoylphosphatidylcholine.