Yeast FIT2 homologs mediate the crosstalk between stress response pathways and cellular proteostasis

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**YEAST *FIT2* HOMOLOGS MEDIATE THE
CROSSTALK BETWEEN STRESS RESPONSE
PATHWAYS AND CELLULAR PROTEOSTASIS**

**PETER TENG SHYU JR.
SCHOOL OF BIOLOGICAL SCIENCES
2019**

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CROSSTALK BETWEEN STRESS RESPONSE
PATHWAYS AND CELLULAR PROTEOSTASIS**

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A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

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Summary

Lipid droplets (LDs) have long been regarded as inert cytoplasmic organelles with the primary function of housing excess intracellular lipids. More recently, LDs have been strongly implicated in conditions of lipid and protein dysregulation. Moreover, these conditions are major contributors to the pathophysiology of metabolic diseases and concomitantly activate cellular stress response pathways, namely the unfolded protein response (UPR) and heat shock response (HSR). However, despite the increasing support for the involvement of LDs in other cellular processes, mechanistic insight into the fundamental process of LD biogenesis and its direct physiological relevance to the cell remains rudimentary. The fat storage inducing transmembrane (FIT) family of proteins comprises of evolutionarily conserved endoplasmic reticulum (ER)-resident proteins that have been reported to induce LD formation. Using *Saccharomyces cerevisiae* as a model, this study aims to dissect the role of LDs in cellular lipid and protein homeostasis through the yeast *FIT* homologs (*ScFIT*), *SCS3* and *YFT2*. While LD biogenesis and basal UPR activation remain unaltered in *ScFIT* mutants, *SCS3* was found to be essential for proper stress-induced UPR activation and for viability in the absence of the sole yeast UPR sensor *IRE1*. Devoid of a functional UPR, *scs3Δ* mutants exhibited accumulation of triacylglycerol within the ER along with aberrant membrane morphology, indicative of impaired ER-LD lipid partitioning and suggesting a UPR-dependent compensatory mechanism for LD maturation. Surprisingly, the absence of the *ScFIT* proteins results in the downregulation of the closely-related HSR pathway. In line with this observation, global protein ubiquitination and the turnover of both ER and cytoplasmic misfolded proteins is impaired in *ScFITΔ* cells, while a screen for interacting partners of *Scs3* identifies components of the proteostatic machinery as putative targets. Taken together, these suggest that *ScFITs* may modulate proteostasis and stress response pathways with lipid metabolism at the interface between the two cellular processes.

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Abbreviations

ATF6	activating transcription factor 6
CDP-Cho	cytidine diphosphate-choline
CDP-DAG	cytidine diphosphate-diacylglycerol
CDP-Etn	cytidine diphosphate-ethanolamine
CoA	coenzyme A
cytoQC	cytoplasmic protein quality control
DG	diacylglycerol
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
FA	fatty acid
FFA	free fatty acid
FIT proteins	fat-storage inducing transmembrane proteins
GC-FID	gas chromatography with flame ionization detector
HSE	heat shock element
HSF1	heat shock factor 1
HSP	heat shock protein
HSR	heat shock response
Ire1 α	inositol-requiring enzyme 1 α
LD	lipid droplet
MG	monoacylglycerol
MYTH	membrane yeast two-hybrid
N-linked	asparagine-linked
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NL	neutral lipid
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PERK	PRKR-like endoplasmic reticulum kinase
PI	phosphatidylinositol
PLIN	perilipin
PL	phospholipid
PS	phosphatidylserine
ScFIT	<i>Saccharomyces cerevisiae</i> FIT

SE	steryl ester
SGA	synthetic genetic array
T2D	type 2 diabetes
TG	triacylglycerol
TLC	thin layer chromatography
Tm	tunicamycin
UPR	unfolded protein response
UPRE	unfolded protein response element
UPS	ubiquitin proteasome system
X-Gal	5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside

Chapter 1. Introduction

1.1 Lipids

Lipids are a class of biomolecules characterised by the presence of fatty acid (FA) constituents, which are carboxylic acids with varying chain length (i.e. number of carbon atoms), branching and degree of unsaturation (i.e. number of double bonds). Different lipid species exist within the cell, and they are as diverse as they are functionally versatile. A wide array of biological processes require lipids for their normal progression, including energy production, membrane biosynthesis, cell signaling, transcriptional regulation and protein modification [1], and failure to maintain cellular lipid homeostasis has been greatly associated with the pathophysiology of various diseases [2, 3]. Based on their inherent structural properties, cellular lipids may broadly be classified as membrane or non-membrane lipids. The former is primarily comprised of amphipathic glycerophospholipids, sphingolipids and sterols, while FAs, neutral lipids and their derivatives are categorised under the latter.

1.1.1 Neutral lipids

Among the different lipid subgroups are the neutral lipids (NL) also referred to as storage lipids, which include triacylglycerols (TG), and sterol esters (SE). NLs are inert, non-polar lipid species that are primarily synthesised in the endoplasmic reticulum (ER) through esterification reactions between FAs and amphipathic alcohols [4]. While several classes of enzymes have diverged through the course of evolution, the process of NL synthesis is essentially and fundamentally conserved down to the unicellular eukaryote, *Saccharomyces cerevisiae* [5]. In yeast cells, TG synthesis is primarily controlled by the activity of two enzymes encoded by *DGA1* (acyl-CoA:diacylglycerol acyltransferase) and *LRO1* (phospholipid:diacylglycerol transferase) [6], and gene products of *ARE1* and *ARE2* (acyl-CoA:sterol acyltransferase) contribute to the synthesis of the yeast SE, ergosteryl ester [7] (Figure 1A). The activity of these enzymes yields TGs that are comprised of three FA chains esterified into a single glycerol backbone and SEs that are formed by esterification of a single FA into the free hydroxyl group of sterols (Figure 1B).

Figure 1. General overview of neutral lipid and phospholipid synthesis in the budding yeast

A) Inert lipid species referred to as neutral lipids are primarily in the form of triacylglycerols (TG) and ergosteryl esters. These are synthesised in the ER from their precursors by the enzymes Dga1, Lro1, Are1 and Are2, and are subsequently stored into lipid droplets (LD, shown in blue). The TG precursors, phosphatidic acid (PA) and diacylglycerol (DG) also serve as important intermediates for the synthesis of a distinct class of amphipathic lipids known as phospholipids. Enzymes that mediate the interconversion of lipid species are indicated in red text. B) Structural diagram of neutral lipids showing three FAs (blue) esterified to a glycerol backbone in TG, and a single FA esterified to a sterol moiety in ergosterol ester. C) Structural diagram of phospholipids with two hydrophobic FAs and the polar head group (green) attached to a phosphor group (red). The or CDP-DAG, cytidine diphosphate-diacylglycerol; CDP-Etn, cytidine diphosphate-ethanolamine; CDP-Cho, cytidine diphosphate-choline; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

Heightened synthesis of NLs has been increasingly associated with various disease states, particularly metabolic syndromes. Dysregulation of cellular TG and SE levels have been reported to influence the progression of both type 2 diabetes (T2D) and non-alcoholic fatty liver disease (NAFLD) [8-13]. However, whether NL synthesis is a contributing factor to the disease state or a part of an adaptive cellular mechanism remains inconclusive. Furthermore, the function of NLs apart from being a storage lipid class remains uncharacterised to this day.

1.1.2 Phospholipids

A close relationship exists between NLs and the synthesis of phospholipids, amphipathic lipid molecules that are the major components of biological membranes. While Dga1 esterifies free FA-coenzyme A (CoA) into diacylglycerol (DG), Lro1 preferentially uses phospholipid-derived acyl groups for DG esterification [6, 14]. Furthermore, the synthesis of NLs shares key intermediates with that of phospholipids either through the *de novo* synthesis pathway or the alternative Kennedy pathway [15] (Figure 1A).

The interconversion of phospholipids is mediated by conserved enzymes and sequentially takes place in different cellular compartments, primarily the ER and the mitochondria. Species diversity among phospholipids is largely accounted for by the headgroups conjugated to their respective phosphate moieties [16-18] (Figure 1C). This structural differentiation provides biophysical properties that then confer distinct functions to each type of phospholipid [19]. With two hydrophobic acyl chains and a hydrophilic headgroup, most phospholipids are constituents of biological membranes. However, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant membrane-forming lipids in most organisms [20]. This generally applies to most organellar membranes, except for the plasma membrane where phosphatidylserine (PS) is predominant. This enrichment of PS is thought to drive endocytic processes [21-24] and apoptosis signaling in higher eukaryotes [25, 26]. Phosphatidylinositol (PI) exhibits a relatively even organellar distribution and functions as a major signaling molecule in various cellular processes through its variable phosphorylation state [27]. Lastly, phosphatidic acid (PA), apart from being a crucial intermediate in phospholipid synthesis additionally regulates cell signaling cascades [28].

1.2 Lipid droplets

1.2.1 Structure and organization of lipid droplets

Due to their highly non-polar nature, NLs are insoluble in the polar cytoplasmic environment and are therefore not structural components of amphipathic cellular membranes. They instead form the core of organelles that arise from the ER known as lipid droplets (LD), otherwise known as lipid bodies or oil bodies. The structure of these dynamic cellular organelles is relatively simple wherein the NL core is surrounded by a phospholipid monolayer, with PC as the major phospholipid component followed by PI and PE [29] (Figure 2). The presence of a monolayer membrane in LDs instead of a bilayer enables the storage of highly hydrophobic NLs. It has been reported that in yeast, SE forms concentric shells beneath the LD membrane and TG lies further within its core. This was hypothesised to confer structural stability as well as a protective function for TG from spontaneous lipolysis [30].

In addition, the phospholipid membrane of LDs selectively houses a distinct set of proteins. The atypical monolayer composition of the LD membrane, as opposed to the bilayer characteristic of other organelles, largely dictates which proteins are able to localise onto its surface. It is hypothesised that the localization of proteins onto LDs is mediated by either an amphipathic helix that is directly inserted into the membrane [31] or through secondary binding interactions with existing proteins on the LD surface [32]. A recent report provides support for an additional regulatory step wherein sizable amphipathic helix of proteins are shown to recognise LD surface regions characterised by membrane packing defects that allow a certain degree of exposure to the NL core underneath [33].

Figure 2. Organization of the yeast lipid droplet

Schematic representations showing A) the structural model of yeast LDs that posit the presence of SE shells enveloping a core of TG beneath the phospholipid monolayer, proteins residing on the LD membrane are depicted in red, and B) the percentage composition of the yeast LD membrane according to lipid species as determined experimentally [29]. PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

The perilipin (PLIN) class of proteins is among the first to be identified as LD-resident proteins [34]. They are primarily expressed in higher eukaryotes where they function in the regulation of stability and lipolytic breakdown of LDs [35-37]. Several high-throughput studies have since then focused on the characterization of the LD proteome in different model organisms. From these reports, an enrichment in enzymes and other factors involved in lipid metabolism were identified, such as NL biosynthetic enzymes and lipases [29, 38-42]. Proteins related to other cellular functions have also been identified, but the physical relevance of their association to LDs has yet to be determined.

1.2.2 Lipid droplet biogenesis, growth and mobilization

While the lipidomic and proteomic aspects of LDs have been extensively characterised, the exact mechanism of LD biogenesis remains obscure. Different models have been proposed [43, 44], but it is agreed upon that the ER is the site of *de novo* LD biogenesis (Figure 3), and the ER localization of all four enzymes required for the synthesis of its NL core strongly support this [6, 7, 14].

Figure 3. Synthesis and breakdown of lipid droplets

Diagram showing the process of LD formation and breakdown. NLs are synthesised by ER-resident enzymes and subsequently accumulate and form a lens between the ER membrane. These then grow towards the cytosol and bud off from the ER. NLs are reversibly broken down into their constituent moieties by the action of lipases and further utilised by the cell for various cellular processes. DG, diacylglycerol; FA, fatty acid; FA-CoA, fatty acid coenzyme-A; MG, monoacylglycerol; PA, phosphatidic acid; PL, phospholipids; TG, triacylglycerol. Figure adapted from [45].

The budding model of LD biogenesis postulates that NLs are synthesised by the ER-resident enzymes and are deposited in between the two leaflets of the ER membrane bilayer. The nucleation of NLs within the phospholipid bilayer leads to the formation of a lens, which upon reaching a size threshold buds off from the ER membrane (Figure 3). Enzymes for the synthesis of NLs alternatively localise on the surface of LDs, presumably through the presence of hydrophobic hairpin domains that mediate its insertion on the phospholipid monolayer [46-49]. From there, the local synthesis of NLs may also drive the growth of existing LDs.

Under conditions of cellular demand, NLs within LDs are mobilised as a source of energy and precursors for other lipid species. This is primarily mediated by triacylglycerol lipases and steryl ester hydrolases, which exert enzymatic activity on TG and SE, respectively [50, 51]. The yeast triacylglycerol lipases, Tgl3/4/5 mediate the hydrolysis of TG into its constituents

DG and free FAs (FFA), which can then be used for energy production or as precursors for the synthesis of membrane phospholipids by series of enzymatic reactions [52, 53]. Similarly, the catabolism of SE into FAs and sterol moieties is catalyzed by the hydrolytic enzymes, Yeh1/2 and Tgl1 [51]. The lipase Tgl4 has also been reported to exhibit steryl ester hydrolase activity [53] (Figure 1A).

Pet10 is a novel PLIN identified in yeast cells that specifically binds to LDs [54]. Transgenic expression of mammalian PLINs in yeast cells similarly led to dynamic ER-LD localization [55]. Interestingly, PLIN expression was found to induce the formation of more LDs in both studies. However, whether this is solely the effect of increased NL synthesis or a synergistic one with protection of LDs from lipolysis is unclear.

1.2.3 Physical and functional relationships between LDs and other organelles

Over the last decade, scientific advancements have provided evidence on the highly dynamic nature of LDs. An increasing body of studies has identified the close physical and functional apposition of LDs to other organelles across different model organisms, suggestive that LDs do not only function as reserves but are also involved in other cellular processes [56, 57]. The fact that most proteins on the LD surface do not exhibit exclusive localization [29] further lends support to the highly interactive nature of LDs [58].

Independent reports have established the intimate association of LDs with different cellular compartments such as the mitochondria [59-61], the vacuole [62-64], the proteasome and the autophagic machinery [65, 66]. The understanding of this intimate association in turn not only provides support for the involvement of LDs in a wide array of non-metabolic cellular processes such as organellar regulation, autophagy and protein quality control [67-70] but also alludes to the potential cascade of cellular damage should LD homeostasis be dysregulated.

1.2.4 Protein effectors of lipid droplet biogenesis

While the appreciation for LDs have grown significantly, definitive insight on the fundamental events that govern its biogenesis remain largely enigmatic to this day. Apart from the initial

step of NL synthesis, whether subsequent processes leading to the formation of mature LDs require the activity of certain proteins or occur spontaneously has been the subject of extensive investigation. There are arguments for the progression of LD formation primarily as a biophysical phenomenon. The nucleation and growth of the NL core within the bilayer could be the result of hydrophobic phase-separation, and the subsequent scission from the ER into the cytoplasm could be governed by the intrinsic properties of the surrounding phospholipid that in turn energetically favor the formation of a droplet structure [71]. Mechanistic studies using the budding yeast as a model organism have alternatively provided invaluable insight into putative proteins factors that regulate the different steps of LD biogenesis.

Yeast *PAH1* encodes for a phosphatidate phosphatase that catalyzes the conversion of PA to DG, the key intermediate for TG synthesis (Figure 1A). Pah1 has also been reported to physically modulate the formation of LDs. The Nem1/Spo7 phosphatase complex regulates Pah1 phosphorylation status and localization to sites of nascent LDs, where it presumably drives LD formation by increasing the rate of TG synthesis [72-74]. Interestingly, while the *pah1Δ* mutant yeast strain has been observed to produce less LDs, the cellular levels of NLs were unaffected and accumulated within the ER [75], thereby raising the possibility that Pah1 may influence LD formation in a manner that is independent from its role in PA-DG conversion. Fld1, the yeast homolog of the human lipodystrophy protein seipin, forms a complex with Ldb16 and functions in the maintenance of ER-LD contact sites [76-78]. A knockout for either one of these proteins caused mutant cells to exhibit aberrantly large LDs, along with a dysregulation in phospholipid biosynthetic genes [79]. In addition, *fld1Δ* mutants exhibited a delay in the formation of LDs [78, 80] and an increase in microsomal PA levels [81]. Similar findings were reported in a separate study wherein changes in the acyl chain composition of phospholipids in *fld1Δ* were hypothesised to drive the formation of large LDs from the fusion of preexisting ones [82]. It was also reported more recently that the expression of the Ldo45 subunit of the Fld1/Ldb16 complex greatly affected the levels of cellular TG, which may yield more direct effects on LD formation [64].

From these, it can be hypothesised that LD formation could be influenced by proteins that either directly regulate NL metabolism or the maintenance of organellar structure, particularly that of the ER. In support of the latter, proteins that regulate physical properties of the ER membrane such as Ice2 and Ubx2 have additionally been linked to in LD biogenesis [83-86].

1.3 Fat storage-inducing transmembrane (FIT) proteins

In addition to the aforementioned proteins, the fat storage-inducing transmembrane (FIT) family of proteins constitutes a group of evolutionarily conserved proteins eponymously named for their role in lipid metabolism and LD formation [87]. The FIT proteins in mammals exhibit differential expression patterns, with FIT1 being expressed primarily in cardiac and skeletal muscle tissue, and FIT2 being more ubiquitously expressed. Topological and biochemical analyses of the murine FIT proteins (FIT1/FIT2) reveal that they are ER-resident proteins with a total of six transmembrane domains and with both N- and C- termini facing the cytosol, wherein the fourth transmembrane domain houses the most highly conserved amino acid residues across different species [87, 88]. Two genes homologous to *FIT2* have been identified in the budding yeast *Saccharomyces cerevisiae* (*ScFIT*), namely *SCS3* and *YFT2*, and are predicted to share the same membrane topology (Figure 4).

Figure 4. The yeast FIT2 homologs (ScFIT), Scs3 and Yft2, share the same topology as the FIT family of proteins

A) Topological model and B) sequence schematic diagram of the ScFIT proteins, Scs3 and Yft2 predict that they are polytopic ER membrane proteins having six transmembrane regions and both C- and N-termini located at the cytosolic side of the ER membrane. Amino acid (aa) residues flanking each of the transmembrane regions are indicated.

1.3.1 Biological functions of FIT proteins

The pioneer study on the FIT proteins has identified its profound effect on the formation and accumulation of LDs both *in vitro* and *in vivo* [87]. Transient overexpression of *FIT2* was sufficient to drive the formation of LDs, a process that was later hypothesised to be mediated by the capacity of FIT2 to directly bind and partition TG from the ER into storage in LDs *in vitro* [88]. Transgenic expression of the mammalian *FIT2* gene in the budding yeast *S. cerevisiae* [89] and in the plant models *Arabidopsis thaliana* and *Nicotiana tabacum* [90] induced the formation of cytoplasmic LDs. Similarly, transient expression of the *ScFIT* genes in mammalian cells *in vitro* led to the increased formation of LDs [89]. A gain-of-function FIT2 mutant was identified with a 3-amino acid mutation within its fourth transmembrane domain, which interestingly houses the most highly-conserved amino acid residues from yeast to humans [91]. Conversely, it was found that the deletion of *FIT2* greatly compromised LD formation in the model organisms, *Danio rerio* and *Caenorhabditis elegans*, as well as in the

pathogenic yeast *Candida parapsilosis* [87, 92, 93]. Similar findings were observed in *FIT2*-ablated human cells cultured *in vitro* [94]. All these reports further reinforced the initial hypothesis of the FIT proteins directly functioning in LD biogenesis.

1.3.2 Functions of the ScFIT proteins, Scs3 and Yft2

In contrast, *S. cerevisiae* deletion mutants for either or both *ScFIT* genes retain their capacity to form LDs, with size and number comparable to those of their WT counterparts [89, 94]. Upon closer investigation, it was found that LDs fail to completely bud off from the ER in the absence of the ScFIT proteins [92], presumably through alterations in ER membrane lipid properties [95]. These findings have since then led to the investigation of alternative functions of the FIT class of proteins. *SCS3* was initially reported to have a putative role in the regulation of phospholipids [96], a function that was largely unexplored until a large-scale genetic screen reported on the strong interactions between the *ScFITs* and genes involved in phospholipid biosynthesis, including *DGK1* and *PSD1* [89]. In-depth analysis of the ScFIT protein sequences have since then revealed the presence of the catalytic site of a lipid phosphatase [97]. This finding was further corroborated by a separate study that reported the same observation on the mammalian FIT2 sequence [94]. *In vitro* analyses have identified the capacity of mammalian FIT2 to hydrolyze phosphates from PA and lyso-PA to yield DAG and MAG, respectively. On the other hand, substrates for the lipid phosphatase activity of the ScFIT proteins have yet to be determined. This conserved catalytic function has been associated with the aberrant ER whorling phenotype observed in cells devoid of the FIT proteins [94, 97].

With these, several different perspectives now exist on the function of the ScFIT proteins. However, definitive evidence for the function of FIT proteins either in binding and partitioning NLs or influencing NL and phospholipid metabolism in the complex *in vivo* environment remains scarce. Moreover, how any of these functions are relevant in the context of cellular function and broader physiological processes has mostly been left uncharacterised.

1.4 Lipid droplets in disease pathophysiology

Mechanistic studies on LDs have been conducted primarily in the unicellular budding yeast as a model organism. While this may be due to the robust genetic tools available for biochemical manipulation in this system, it also alludes to the increasing complexity of LD biogenesis in higher eukaryotes. Interestingly, mutations in proteins associated with LD formation in *S. cerevisiae* yield modest phenotypes under normal physiological conditions, and yet give rise to gross and more severe defects in higher organisms with the corresponding genetic background. For example, deletion of seipin (*SEI1*) in yeast caused a delay in *de novo* LD formation and aberrant LD morphology, but otherwise yielded minimal effect on cell growth [80]. However, human seipin, also known as the Berardinelli-Seip congenital lipodystrophy type 2 protein, was found to not only be critical for adipocyte differentiation and lipogenesis induction using *in vitro* cell cultures [98], but is linked to a more severe form of congenital general lipodystrophy characterised by insulin resistance, hepatic steatosis and extreme reduction in both metabolically-active and mechanical adipose tissue in patient studies [99]. Similarly, loss of the yeast *FIT2* gene homologs impaired LD maturation without affecting general cellular fitness, but a corresponding loss of homologs resulted in early death in both the nematode *Caenorhabditis elegans* and mouse models [92, 100]. All these lend support to the role of LDs in both organismal development and metabolic disease predisposition.

Following this paradigm, the ability to form and regulate the formation of LDs intuitively confers a cytoprotective effect. The synthesis of NLs and their subsequent compartmentalization into LDs may serve to buffer against atypical accumulation of and chronic exposure to highly reactive FFAs, which could otherwise lead to cellular dysfunction and ultimately cell death in a process termed as lipotoxicity. Several diseases states have been attributed to lipotoxicity [101], including NAFLD and T2D. FFAs have been reported to activate the NF- κ B inflammatory pathway and induce the expression of the pro-inflammatory cytokine tumor necrosis factor- α in a mouse model of non-alcoholic steatohepatitis (NASH) [102]. It was also found that elevated levels of FFAs resulted in insulin-resistance in mice [103], and induced pancreatic β -cell death *in vitro* [104], thus directly contributing to two hallmark characteristics of the T2D.

In yeast, a *pah1* Δ mutant deficient for the enzyme needed for the conversion of PA to DG exhibited increased levels of intracellular FFAs, sensitivity to exogenous FA exposure, and apoptotic characteristics upon entry to stationary phase [105]. A similar phenotype was found in *dga1* Δ *lro1* Δ *are1* Δ *are2* Δ cells that are incapable of NL synthesis [106, 107]. Furthermore, an *in vitro* study using pancreatic β -cells has reported that TG formation resulting from unsaturated fatty acid supplementation confers a cytoprotective effect upon prolonged exposure to saturated fatty acids, which is otherwise lipotoxic and leads to apoptosis [108]. SFAs have been reported to exert lipotoxic effects by compromising the capacity of cells to synthesise and store NLs into LDs [109]. This further suggests that the subsequent storage of synthesised NLs into LDs constitutes a critical step in the prevention of lipotoxicity, and that LDs may protect NLs against exposure to spontaneous enzymatic activity. Consistent with this, the presence of LDs in glial cells were found to prevent oxidation of polyunsaturated fatty acids, thereby protecting neuroblasts from damage caused by reactive oxygen species [110].

Dysregulation of phospholipids is also characteristic of certain cardiovascular and neurodegenerative diseases. Disruption of PE synthesis in *Drosophila* reportedly increased TG levels, associated with impaired cardiac function [111, 112]. Disruption of PC synthesis in a yeast model of NASH also led to the significant increase in TG and LD levels [113]. Conversely, PC synthesis has been reported to directly influence both LD size and NL synthesis [114]. Collectively, these highlight the interdependence of NLs and phospholipids and further suggest that LDs could regulate levels of phospholipids by either supplying or sequestering intermediates, thereby preventing cellular dysfunction caused by gross alterations in the cellular phospholipid profile.

On the other hand, the aberrant accumulation of LDs has been identified in various disease states, indicating that an intricate balance in maintaining the amount of cellular LDs is critical for cellular homeostasis [115, 116]. Therefore, it could not be dismissed that the presence of LDs, when dysregulated, may potentially drive disease progression. The aggregation-prone α -synuclein protein, which is a causative factor in the development of Parkinson's disease, has been reported to bind onto the LD membrane surface and additionally impair the lipolytic

breakdown of LDs [117, 118]. In connection to this, pharmacological inhibition of sterol ester synthesis decreased the production of amyloid- β , a proteotoxic protein relevant in Huntington's disease pathology [119, 120]. From these, it can be argued that the phenotypes associated with neuropathies could additionally be the result of dysregulated LD homeostasis. The hepatitis C virus has also been reported to rely on the presence of LDs for its assembly and replication [121-123]. Lastly, increased levels of cytoplasmic LDs have been thought to drive the aggressiveness and proliferative capacity of several cancer cell lines [124, 125].

1.5 The unfolded protein response (UPR)

Studies have described the close association between LDs and the ER on both the physical and functional levels [46, 76, 126], which is further supported by the consensus that LD biogenesis is initiated in the ER. The ER is an extensive membrane network that coordinates multiple cellular processes through its connection with various organelles [127]. Perhaps the most critical among these are the synthesis and processing of cellular lipids and proteins.

Early work in mammalian and yeast cells have reported on how the genetic or pharmacological disruption of proper ER homeostasis induced the expression of the ER luminal protein chaperone BiP/Kar2 [128, 129]. It was later described that the presence of misfolded proteins caused ER stress and the subsequent upregulation of gene expression through a 22-base pair element in the promoter region [130, 131]. This promoter sequence was then termed as the unfolded protein response (UPR) element [131]. Since then, several studies have established the UPR as a signal transduction pathway that is initiated in the ER and is activated under conditions of protein stress. In recent years, it has been discovered that the UPR is also activated from gross insults to the lipidomic landscape of the cell, such as membrane phospholipid perturbation and deletion of certain genes in the different lipid metabolic pathways [113, 132-135].

1.5.1 UPR activation mechanism

The UPR is an adaptive response pathway that aims to restore ER homeostasis by modulating the expression of downstream target genes, and alternatively drives pro-apoptotic

pathways should the stress condition remain unresolved. In metazoans, the UPR is mediated by signalling cascade events effected by three distinct ER transmembrane proteins: inositol-requiring enzyme 1 α (Ire1 α), PRKR-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6). Among these, the most evolutionarily conserved and well-studied from yeast to humans being the Ire1 axis (Figure 5).

Figure 5. The unfolded protein response signal transduction pathway

Conditions of ER stress (i.e. presence of unfolded proteins and lipid perturbation) activate the UPR signaling pathways in cells. In metazoans, stressors are affected by three distinct axes, namely Ire1 α , PERK and ATF6. The cognate transcription factors, Xbp1, ATF4 and ATF6(N), then translocate into the nucleus to modulate gene expression including those of ER luminal chaperones and ER-associated degradation (ERAD) components. UPR activation concurrently attenuates global protein translation and induces LD formation to restore homeostasis. Failure to resolve the stress condition initiates apoptosis in higher eukaryotes. The evolutionarily conserved Ire1 pathway in *Saccharomyces cerevisiae* and its downstream Hac1 effector (Xbp1 in metazoans) are highlighted in blue. Figure adapted from [45].

In the budding yeast, UPR activation and signal transduction is dependent on the presence of the integral ER membrane protein, Ire1. The luminal domain of Ire1 acts as a sensor for the presence of misfolded proteins within the ER, while lipid and membrane aberrancy is reportedly sensed by its transmembrane domain. Either of these stressors culminates in the oligomerisation and activation of Ire1, thereby allowing its cytoplasmic endonuclease domain to cleave an inhibitory intron from the pre-mRNA of *HAC1*, which is necessary for its subsequent translation. Mature Hac1 then translocates into the nucleus where it recognises the UPR element (UPRE) in promoter regions, thereby leading to the transcription of approximately 381 UPR target genes to restore cellular homeostasis [136-138] (Figure 5). These genes include those involved in protein folding and ER-associated degradation (ERAD), lipid metabolism and vesicular trafficking among others [137].

1.6 The heat shock response (HSR)

A closely related stress response pathway to the UPR is that of the heat shock response (HSR). Eponymously identified to be triggered by heat stress, the HSR is also activated under conditions of cytoplasmic misfolded protein accumulation [139-141]. In brief, recognition of the stress stimulus activates the master transcriptional regulator of the HSR, heat shock factor 1 (HSF1), which then upregulates the expression of proteins to promote protein folding and turnover [142]. These include subunits of the proteasomal degradation machinery and molecular chaperones with their cognate partners termed as heat shock proteins (HSP) that aid in the maturation, folding, and refolding of client proteins [143, 144].

1.6.1 HSR activation mechanism

In higher eukaryotes, the presence of stress stimulus (i.e. heat, cytoplasmic misfolded proteins) causes the monomeric HSF1 sensor to dissociate from a repressive chaperone complex and subsequently form a homotrimeric transcription factor [145]. This homotrimer is then phosphorylated before translocating into the nucleus to modulate the expression of its target genes [146] (Figure 6). Hsf1 is similarly required for HSR activation in the budding yeast but is also essential under normal physiological conditions [147]. Consistent with its essential nature, Hsf1 regulates the expression of genes that are involved in a broad range of cellular

processes including energy metabolism and structural integrity of cells. This is achieved through the binding of the transcription factor onto heat shock elements (HSE) in promoter regions of target genes [148]. Furthermore, yeast Hsf1 is constitutively trimeric, nuclear and bound to its targets, in stark contrast to the dynamic cytoplasmic-nuclear localization of HSF1 in metazoans [149]. The extent of HSR activation is then regulated not through its localization, but rather through post-translational modification within the nucleus itself. It was found that the heat stress induced the hyperphosphorylation of Hsf1, which correlates to the transcription of its target genes [150, 151]. While the exact molecular events that lead to yeast phosphorylation is not fully understood, the Rim15, Yak1 and Snf1 kinases have been implicated in this process [152-154] (Figure 6).

Figure 6. The heat shock response signal transduction pathway

The presence of misfolded proteins in the cytoplasm and elevated, non-physiological temperatures activate the HSR. Under these conditions, monomeric HSF1 in metazoans undergoes phosphorylation and trimerization to give rise to the functional HSF1 transcription factor. The HSR in *Saccharomyces cerevisiae* showing the constitutive nuclear localization of the Hsf1 transcriptional regulator is highlighted in green. Figure adapted from [45].

1.7 Lipid droplets and cell stress response regulation

While there are variations in the intensity of UPR activation as well as differential regulation of downstream target genes dependent on the cause of stress [155-158], both protein and lipid-induced UPR activation similarly result in increased lipogenic markers and subsequently LD formation [113, 159-161]. Interestingly, mutants that are incapable of LD formation conversely upregulated the UPR, thus strongly indicative of a role for LDs under the UPR program. However, the dispensability of NL synthesis for viability under ER stress conditions [159] suggests that the constituent LD core may not be the sole contributor to the homeostatic response and that LDs have another function in protein-induced ER stress. Exposure to chronic heat stress similarly increased the synthesis of TG and the formation of LDs in fission and budding yeast, respectively [162, 163], and the prominent downstream Hsp70 effector of the HSR was also reported to drive lipogenic pathways in HepG2 cells and increase intracellular LDs [164]. However, the rationale for the upregulation of LDs under these stress conditions remains a topic of extensive scientific debate.

1.8 Cellular proteostatic machineries

1.8.1 ERAD and the ubiquitin proteasome system (UPS)

Cellular proteins are primarily synthesised in the ER, where they undergo extensive post-translational modifications through the secretory pathway before being processed and transported to their respective cellular compartments. However, the process of protein synthesis is not entirely infallible, and as such proteins may occasionally adopt non-native conformations *en route* to maturation. In these situations, molecular chaperones often bind to the hydrophobic patches that are aberrantly exposed in misfolded proteins to maintain their solubility within the ER lumen. Several classes of chaperones, also referred to as HSPs, are expressed by cells and are widespread across different cellular compartments, each playing a distinct role in the binding and refolding of client proteins [165].

One of the key post-translational modifications that proteins are subjected to as they fold is the attachment of oligosaccharide/glycan molecules to key amino acid residues, and this process is called asparagine-linked (*N*-linked) glycosylation. The variable branched state of these

auxiliary glycan groups serves as a crucial molecular signal that determines the fate of nascent proteins within the ER [166]. These proteins may undergo a series of folding through the action of resident chaperones, or alternatively persist as terminally-misfolded proteins. As the accumulation of non-native proteins can be detrimental not only to ER function but also cell viability, such proteins that fail to fold correctly within the ER are retained and targeted towards destruction through ERAD, the terminal phase of ER protein quality control [167] (Figure 7).

Figure 7. The Doa10 and Hrd1 complexes are the two main E3 ligases for ER-associated degradation in the budding yeast

Misfolded proteins in the ER are marked for degradation through ubiquitination by the Doa10 and Hrd1 multi-subunit complexes. Cytosolic lesions in client substrates are recognised by the Doa10 complex (ERAD-C) while lesions in the transmembrane domain of ER integral proteins and misfolded ER luminal proteins are recognised by the Hrd1 complex (ERAD-M, ERAD-L). Figure adapted from [167] with permission.

Under ERAD, proteins are conventionally marked for degradation via the attachment of ubiquitin molecules to lysine residues on the target protein, although other amino acid residues have additionally been reported to be ubiquitinated for ERAD [168]. This occurs through the sequential catalytic activity of ubiquitin-activating (E1), ubiquitin-conjugating (E2) enzymes, and the ubiquitin ligases (E3). These E3 ubiquitin ligases exist as multi-subunit complexes on the ER membrane and are collectively referred to as either the Doa10 or the Hrd1 complexes, after the E3 ligase that mediates the final step of ubiquitin linkage to proteins

(Figure 7). These two complexes canonically exhibit a specificity towards their client proteins. Doa10 recognises misfolded proteins through cytosolic defects (ERAD-C for cytosol), while Hrd1 ubiquitinates proteins with aberrant conformations in their transmembrane domain (ERAD-M for transmembrane) or those within the lumen of the ER (ERAD-L for lumen) [167]. Finally, after proteins are ubiquitinated, they are retrotranslocated into the cytosol via the Cdc48 ATPase, where they are routed for degradation through enzymatic cleavage via the ubiquitin proteasome system (UPS) (Figure 8). Finally, it is noteworthy that ERAD not only is critical for the degradation of potentially toxic misfolded proteins but is equally essential for the natural turnover of native proteins under normal physiological conditions [47, 169].

Figure 8. Terminally-misfolded proteins are routed to the proteasome for degradation

Following ubiquitination by the ERAD ligases, misfolded ER proteins are retrotranslocated into the cytoplasm and degraded in the cytoplasmic proteasome. Substrates of the nuclear San1 E3 ubiquitin ligase through cytoplasmic protein quality control (cytoQC) are similarly processed in the proteasome. Figure adapted from [167] with permission.

1.8.2 Cytosolic protein quality control (cytoQC)

While properly folded proteins exit the secretory pathway and eventually localise to their corresponding compartments, their conformation may be challenged by stress stimuli such as heat and oxidative agents [153, 170]. To maintain proteostasis, several quality control checkpoints are in place in different cellular compartments. In the cytoplasm, the degradation of misfolded proteins may proceed through autophagy [171] or alternatively through a separate branch of the UPS termed as the cytoplasmic protein quality control (cytoQC) checkpoint. Under cytoQC, proteins are similarly ubiquitinated as they are in ERAD. However, this process is mediated by a distinct class of E3 ubiquitin ligases. The nuclear San1 E3 ubiquitin ligase is the major effector of ubiquitination both in the cytoplasm and in the nucleus [172, 173]. While the San1-dependent degradation of proteins within the nucleus proceeds intuitively due to their similar localization, cytoplasmic proteins employ the extranuclear chaperones, Ydj1 and Sse1, and the E3 ligase Ubr1 for import into the nucleus and subsequent ubiquitination [174, 175]. This is then succeeded by proteasomal degradation, but it remains unclear whether these ubiquitinated proteins are shuttled back into the cytoplasm for this process or are retained within the nucleus for intraorganellar degradation through nuclear proteasomes [176] (Figure 8).

1.8.3 Lipid droplets and proteostasis

While the ablation of LD formation by genetic or pharmacological disruption of NL synthesis is dispensable for ERAD function in both yeast and mammalian cell models [177, 178], a growing body of reports have reported on the involvement of LDs in cellular proteostasis. The ERAD-associated protein AUP1 that mediates substrate ubiquitination has both been found to localise to the LD membrane and to regulate LD abundance [179]. A potential function of LDs in response to protein stress is by serving as temporary depot for proteins destined for degradation [48]. In line with this, HMG-CoA, the rate-limiting enzyme in cholesterol biosynthesis, is shuttled onto LDs as part of its endogenous degradation mechanism [169]. Similarly, ubiquitinated apoB localises to LDs at sites in contact with the proteasome and is further supported by co-fractionation of LDs with ubiquitinated apoB, and 20S proteasomal subunits [65]. From these, it may be hypothesised that apart from its NL core, the LD

phospholipid membrane may serve a proteostatic function, specifically by housing client proteins that are marked for degradation. Additionally, the contribution of LDs in the maintenance of proper protein turnover may be related to its upregulation by the UPR and HSR stress response pathways.

1.9 Aims of this study

The strong association of dyslipidemia and the UPR with the progression of metabolic diseases warrant the investigation of how these two contributing factors may be related to each other. While the function of the FIT proteins in relation to LD biogenesis is well characterised, its relevance to the UPR is largely unknown. In this study, we investigated the role of the yeast *FIT2* homolog *SCS3*, identified as one of the downstream UPR target genes, in lipid metabolism and the two closely related cellular stress responses, namely the UPR and the HSR. We take advantage of the robust tools for the investigation of the yeast UPR, which is solely dependent on the *IRE1*-mediated signal transduction pathway. This allows ease of manipulation for titrating phenotypes that may contribute to this cell response mechanism. Furthermore, the gross physiological defects associated with FIT aberrations in complex multicellular organisms warrant the investigation of specific cellular processes that are directly affected in the absence of FIT proteins within the context of cell stress response regulation.

Chapter 2. Materials and methods

2.1 *Saccharomyces cerevisiae* strains and genetic recombination methods

S. cerevisiae strains used in this study are listed in Table 1. Transformation of yeast strains was conducted using the lithium acetate (LiOAc) method as previously described [180]. In brief, activated strains were grown to 0.6-0.8 OD₆₀₀/ml in 10 ml of the appropriate culture media. Cells were pelleted at 1000 ×g, washed and made competent by resuspension in LiOAc mix (100 mM LiOAc, 10 mM Tris-HCl pH 7.4, 1 mM EDTA). The cell suspension was then added to a solution of PLATE mix (40% PEG 3350, 100 mM LiOAc, 10 mM Tris-HCl pH 7.4, 1 mM EDTA), ssDNA and gene product before incubation at 30°C for one hour. The transformation reaction was then added with DMSO and subjected to heat shock at 42°C for 20 min. Afterwards, cells were allowed to recover before plating onto the appropriate selection media. Successful transformants were selected and verified after incubation in 30°C for 48-72 h.

Genetic crossing was carried out by mixing two diploid strains of complementary mating type on an agar plate and incubation in 30°C for four hours. Zygotes were picked and sporulated for five days in room temperature (RT). Afterwards, tetrads were picked using the Sporeplay Modular Microscope Platform (Singer Instruments).

Table 1. Yeast strains used in this study

Strain	Genotype	Source
W303	<i>MATa, leu2-3, -112, his3-11, trp1-1, ura3-1, can1-100, ade2-1</i>	[181]
PER	<i>MATa, ire1::TRP1, ura3-1, can1-100, ade2-1, ade3, leu2-3-112, his3-11::HIS3-UPR-LacZ</i>	[182]
NMY51	<i>MATa, his3Δ200, trp-901, leu2-3-112, ade2, LYS::(lexAop)4-HIS3, ura3::(lexAop)8-LacZ, (lexAop)8-ADE2, GAL4</i>	[183]
YGT0028	<i>MATa, pJC31, W303 background</i>	[113]
YGT0038	<i>MATa, cho2::KanMX, W303 background</i>	[113]
YGT0050	<i>MATa, W303 background</i>	[113]
YGT0112	<i>MATa, ire1::KanMX, W303 background</i>	[113]
YGT0114	<i>MATa, ire1::KanMX, pJC31, W303 background</i>	[113]
YGT0306	<i>MATa, pGT0181, W303 background</i>	[184]
YGT0381	<i>MATa, dga1::KanMX, lro1::KanMX, are1::KanMX, are2::KanMX, his3Δ, leu2Δ0, lys2Δ0, ura3Δ0, BY4742 background</i>	[107]
YGT0492	<i>MATa, ire1::TRP1, yft2::KanMX, pDN388, PER background</i>	This study
YGT0507	<i>MATa, scs3::KanMX, W303 background</i>	This study
YGT0508	<i>MATa, yft2::KanMX, W303 background</i>	This study
YGT0518	<i>MATa, ire1::TRP1, scs3::KanMX, pDN388, PER background</i>	This study

Strain	Genotype	Source
YGT0529	<i>MATa</i> , <i>scs3::KanMX</i> , pJC31, W303 background	This study
YGT0530	<i>MATa</i> , <i>yft2::KanMX</i> , pJC31, W303 background	This study
YGT0536	<i>MATa</i> , <i>psd1::KanMX</i> , W303 background	This study
YGT0542	<i>MATa</i> , <i>scs3::KanMX</i> , <i>yft2::KanMX</i> , W303 background	This study
YGT0543	<i>MATa</i> , <i>scs3::KanMX</i> , <i>yft2::KanMX</i> , pJC31, W303 background	This study
YGT0561	<i>MATa</i> , pGT0227, W303 background	This study
YGT0562	<i>MATa</i> , <i>scs3::KanMX</i> , pGT0227, W303 background	This study
YGT0563	<i>MATa</i> , <i>yft2::KanMX</i> , pGT0227, W303 background	This study
YGT0564	<i>MATa</i> , <i>scs3::KanMX</i> , <i>yft2::KanMX</i> , pGT0227, W303 background	This study
YGT0565	<i>MATa</i> , pGT0228, W303 background	This study
YGT0660	<i>MATa</i> , <i>ire1::TRP1</i> , <i>scs3::KanMX</i> , pDN388, pGT0286, PER background	This study
YGT0661	<i>MATa</i> , <i>ire1::TRP1</i> , <i>scs3::KanMX</i> , pGT0003, pGT0286, PER background	This study
YGT0662	<i>MATa</i> , <i>ire1::TRP1</i> , <i>scs3::KanMX</i> , pDN388, pGT0004, PER background	This study
YGT0663	<i>MATa</i> , <i>ire1::TRP1</i> , <i>scs3::KanMX</i> , pDN388, pGT0364, PER background	This study
YGT0664	<i>MATa</i> , <i>ire1::TRP1</i> , <i>scs3::KanMX</i> , pGT0003, pGT0364, PER background	This study
YGT0666	<i>MATa</i> , <i>SCS3-GFP::HIS3MX6</i> , <i>RTN1-yomRuby2::KanMX</i> , W303 background	This study
YGT0667	<i>MATa</i> , <i>YFT2-GFP::HIS3MX6</i> , <i>RTN1-yomRuby2::KanMX</i> , W303 background	This study
YGT0670	<i>MATa</i> , pGT0374, NMY51 background	This study
YGT0762	<i>MATa</i> , pGT0363, NMY51 background	This study
YGT0764	<i>MATa</i> , <i>ept1::KanMX</i> , W303 background	This study
YGT0814	<i>MATa</i> , pGT0374, pPM28, NMY51 background	This study
YGT0857	<i>MATa</i> , <i>scs3::KanMX</i> , <i>psd1::KanMX</i> , W303 background	This study
YGT0858	<i>MATa</i> , <i>P_{TDH3}-SCS3::KanMX</i> , W303 background	This study
YGT0869	<i>MATa</i> , <i>psd1::KanMX</i> , pJC31, W303 background	This study
YGT0870	<i>MATa</i> , <i>scs3::KanMX</i> , <i>psd1::KanMX</i> , pJC31, W303 background	This study
YGT0916	<i>MATa</i> , <i>scs3::KanMX</i> , <i>ept1::KanMX</i> , W303 background	This study
YGT0925	<i>MATa</i> , <i>ept1::KanMX</i> , pJC31, W303 background	This study
YGT0926	<i>MATa</i> , <i>scs3::KanMX</i> , <i>ept1::KanMX</i> , pJC31, W303 background	This study
YGT0954	<i>MATa</i> , <i>ice2::KanMX</i> , W303 background	This study
YGT0959	<i>MATa</i> , pRP42, W303 background	This study
YGT0960	<i>MATa</i> , <i>scs3::KanMX</i> , pRP42, W303 background	This study
YGT0961	<i>MATa</i> , <i>yft2::KanMX</i> , pRP42, W303 background	This study
YGT0962	<i>MATa</i> , <i>scs3::KanMX</i> , <i>yft2::KanMX</i> , pRP42, W303 background	This study
YGT0963	<i>MATa</i> , pRP44, W303 background	This study
YGT0964	<i>MATa</i> , <i>scs3::KanMX</i> , pRP44, W303 background	This study
YGT0965	<i>MATa</i> , <i>yft2::KanMX</i> , pRP44, W303 background	This study
YGT0966	<i>MATa</i> , <i>scs3::KanMX</i> , <i>yft2::KanMX</i> , pRP44, W303 background	This study
YGT0967	<i>MATa</i> , <i>scs3::KanMX</i> , <i>ice2::KanMX</i> , W303 background	This study
YGT0972	<i>MATa</i> , <i>ice2::KanMX</i> , pJC31, W303 background	This study
YGT0973	<i>MATa</i> , <i>scs3::KanMX</i> , <i>ice2::KanMX</i> , pJC31, W303 background	This study
YGT1034	<i>MATa</i> , pGT0428, NMY51 background	This study
YGT1035	<i>MATa</i> , pGT0427, NMY51 background	This study
YGT1036	<i>MATa</i> , pGT0426, NMY51 background	This study
YGT1046	<i>MATa</i> , pGT0235, W303 background	This study
YGT1047	<i>MATa</i> , <i>scs3::KanMX</i> , pGT0235, W303 background	This study
YGT1048	<i>MATa</i> , <i>yft2::KanMX</i> , pGT0235, W303 background	This study
YGT1049	<i>MATa</i> , <i>scs3::KanMX</i> , <i>yft2::KanMX</i> , pGT0235, W303 background	This study
YGT1050	<i>MATa</i> , pGT0428, pPM28, NMY51 background	This study
YGT1051	<i>MATa</i> , pGT0427, pPM28, NMY51 background	This study
YGT1052	<i>MATa</i> , pGT0426, pPM28, NMY51 background	This study
YGT1069	<i>MATa</i> , pGT0246, W303 background	This study

Strain	Genotype	Source
YGT1070	<i>MATa</i> , <i>scs3::KanMX</i> , pGT0246, W303 background	This study
YGT1071	<i>MATa</i> , <i>yft2::KanMX</i> , pGT0246, W303 background	This study
YGT1072	<i>MATa</i> , <i>scs3::KanMX</i> , <i>yft2::KanMX</i> , pGT0246, W303 background	This study
YGT1074	<i>MATa</i> , pPK249, W303 background	This study
YGT1075	<i>MATa</i> , <i>scs3::KanMX</i> , pPK249, W303 background	This study
YGT1076	<i>MATa</i> , <i>yft2::KanMX</i> , pPK249, W303 background	This study
YGT1077	<i>MATa</i> , <i>scs3::KanMX</i> , <i>yft2::KanMX</i> , pPK249, W303 background	This study
YGT1078	<i>MATa</i> , pPC1040, W303 background	This study
YGT1079	<i>MATa</i> , <i>scs3::KanMX</i> , pPC1040, W303 background	This study
YGT1080	<i>MATa</i> , <i>yft2::KanMX</i> , pPC1040, W303 background	This study
YGT1081	<i>MATa</i> , <i>scs3::KanMX</i> , <i>yft2::KanMX</i> , pPC1040, W303 background	This study
YGT1082	<i>MATa</i> , pPC1196, W303 background	This study
YGT1083	<i>MATa</i> , <i>scs3::KanMX</i> , pPC1196, W303 background	This study
YGT1084	<i>MATa</i> , <i>yft2::KanMX</i> , pPC1196, W303 background	This study
YGT1085	<i>MATa</i> , <i>scs3::KanMX</i> , <i>yft2::KanMX</i> , pPC1196, W303 background	This study
YGT1086	<i>MATa</i> , pPC1299, W303 background	This study
YGT1087	<i>MATa</i> , <i>scs3::KanMX</i> , pPC1299, W303 background	This study
YGT1088	<i>MATa</i> , <i>yft2::KanMX</i> , pPC1299, W303 background	This study
YGT1089	<i>MATa</i> , <i>scs3::KanMX</i> , <i>yft2::KanMX</i> , pPC1299, W303 background	This study
YGT1090	<i>MATa</i> , STK05-1-2, W303 background	This study
YGT1091	<i>MATa</i> , <i>scs3::KanMX</i> , STK05-1-2, W303 background	This study
YGT1092	<i>MATa</i> , <i>yft2::KanMX</i> , STK05-1-2, W303 background	This study
YGT1093	<i>MATa</i> , <i>scs3::KanMX</i> , <i>yft2::KanMX</i> , STK05-1-2, W303 background	This study
YGT1178	<i>MATa</i> , <i>scs3::KanMX</i> , <i>yft2::KanMX</i> , pGT0181, W303 background	This study

2.2 Plasmids and antibodies

Plasmids and oligonucleotide primers used in this study are detailed in Table 2 and 3, respectively. Plasmids constructs were generated through either conventional restriction enzyme cloning methods or Gibson Assembly (New England Biolabs). The mutant *scs3* library was generated by low-fidelity PCR using primers PS1-PS2 to amplify the promoter, coding sequence, and terminator regions of the *SCS3* from the genomic DNA of wild type (WT) cells. The PCR product was then digested with the enzymes *EcoRI* and *XbaI* before ligation into pGT0004. Plasmid pGT0364 was obtained through a colony sectoring screen detailed in Section 2.8. Plasmid pGT0286 encoding for WT *SCS3* was similarly generated using conventional PCR amplification. To generate reporter constructs for the membrane yeast two hybrid screen, the coding sequences of *SCS3* and *YFT2* were amplified from WT yeast DNA using primer pairs PS39-PS40 and PS159-PS160, respectively. These were then inserted via Gibson Assembly into vector backbones generated through PCR from pGT0317 using primer pairs PS39-PS40 and PS157-PS158, respectively, to generate pGT0374 and pGT0427. Plasmids pGT0426 and pGT0428 were generated through Gibson Assembly by amplifying the coding sequence of *SCS3* and *YFT2* terminating immediately before the stop codon using WT

yeast DNA with primer pairs PS107-PS108 and PS101-PS102, respectively. These were then cloned into PCR-amplified vector backbones using pGT0318 as template with primer pairs PS105-PS106 and PS36-PS99, respectively.

Table 2. Plasmids used in this study

Plasmid	Encoded protein	Promoter	Vector	Source
pDN388	Ire1/Ade3	<i>IRE1/ADE3</i>	pRS315	[182]
pJC31	β -galactosidase (LacZ)	<i>UPRE-CYC1</i>	pRS314	[181]
pPC1040	3HA-Pgc1	<i>PGC1</i>	pRS315	[47]
pPC1196	Dga1-GFP	<i>ADH1</i>	pRS415	[47]
pPC1299	Yeh1-3HA	<i>YEH1</i>	pRS316	[47]
pPK249	ngPrA* Δ 295–331-HA	<i>PEP4</i>	pRS316	[185]
pPM28	eroGFP	<i>GAP</i>	pRS316	[186]
pRP42	Δ ssPrA-HA	<i>TDH3</i>	pRS313	[173]
pRP44	Δ 2GFP	<i>TDH3</i>	pRS313	[173]
pGT0003	-	-	pRS315	[187]
pGT0004	-	-	pRS316	[187]
pGT0181	Cue1-HA	<i>CUE1</i>	pRS315	[184]
pGT0227	β -galactosidase (LacZ)	<i>HSE-SSA3</i>	pCM64	[188]
pGT0228	β -galactosidase (LacZ)	<i>mHSE-SSA3</i>	pCM64	[188]
pGT0235	Ub-myc	<i>CUP1</i>	YEpl05	[189]
pGT0246	CPY*-HA	<i>PRC1</i>	pRS316	[158]
pGT0286	Scs3	<i>SCS3</i>	pRS316	This study
pGT0317	-	<i>TEF1</i>	pTLB1	[190]
pGT0318	-	<i>TEF1</i>	pTMBV α	[190]
pGT0363	CD4 transmembrane domain	<i>ADH1</i>	pAMBV	[191]
pGT0364	scs3-1	<i>SCS3</i>	pRS316	This study
pGT0374	(LexA-VP16-C _{ub})-Scs3	<i>TEF1</i>	pTLB-1	This study
pGT0426	Yft2-(C _{ub} -LexA-VP16)	<i>TEF1</i>	pTMBV α	This study
pGT0427	(LexA-VP16-C _{ub})-Yft2	<i>TEF1</i>	pTLB1	This study
pGT0428	Scs3-(C _{ub} -LexA-VP16)	<i>TEF1</i>	pTMBV α	This study
STK05-1-2	HA-Sbh2	<i>MET25</i>	p413MET25	[192]

Table 3. Oligonucleotide primers used in this study

Primer	Sequence (5' to 3')
PS1	GATCGCCGACTCCATGAACC
PS2	GGAGCAAGGCAAACCTACACG
PS39	TCCTGCAGATATACCCATG
PS40	CGACAAAGCTGATCTGTG
PS41	TGGTATGCACAGATCAGCTTTGTCTGATGTCTAGCAAATGGTTTAAATG
PS42	GGCCTCCATGGGTATATCTGCAGGATCATACTGGACGTAGCGC
PS99	AAAGGCCTCCATGGGTATATC
PS101	CTTTGGATAAAAGAGCCATGATACGTCAGCTCAATTATTG
PS102	CCCATGGAGGCCTTTTAGATATAAGTAATTGTGTAGTATCCCAATTT
PS105	TATATCTGCAGGAATTCTG
PS106	GGCTCTTTTATCCAAAGATAC
PS107	CTTTGGATAAAAGAGCCATGTCTAGCAAATGGTTTAAATGCTATACAC
PS108	CGAATTCCTGCAGATATATACTGGACGTAGCGCGGC
PS157	AATTCCTGCAGATATACCCATG
PS158	CGTCGACAAAGCTGATCTG
PS159	ACAGATCAGCTTTGTCTGACGATGATACGTCAGCTCAATTATTG
PS160	TGGGTATATCTGCAGGAATTTTCATAGATATAAGTAATTGTGTAGTATCC

Polyclonal rabbit antibodies against Kar2 were generously provided by Davis Ng (Temasek Life Sciences Laboratories, Singapore). The monoclonal mouse α -HA (Covance), polyclonal rabbit α -LexA (Abcam), mouse monoclonal α -c-Myc (Invitrogen), mouse monoclonal α -tubulin (DSHB) and mouse monoclonal α -GFP (Roche) primary antibodies were commercially purchased. Goat-derived secondary antibodies α -rabbit IgG-DyLight 550 (Invitrogen), α -mouse IgG-DyLight 488 (Invitrogen) α -mouse IgG (H+L) Alexa Fluor 488 (Invitrogen), α -mouse IgG-IRDye 800 (LI-COR) and α -rabbit IgG-IRDye 680 were similarly purchased.

2.3 Yeast growth assays

Overnight activation cultures of yeast strains grown to saturation were diluted to $OD_{600}=0.2/\text{ml}$, from which three 10-fold serial dilutions were prepared. The cell suspensions were then spotted onto appropriate agar plates and incubated in indicated temperatures for 72 h or until colonies are seen in the final dilution.

2.4 Protein extraction and immunoblotting

For yeast protein extraction, two OD units of cells were typically pelleted and resuspended in 1 ml of 10% trichloroacetic acid (TCA, Sigma). Cells were lysed with two rounds of mechanical disruption using silica beads at 6500 rpm for 30 s using the Precellys 24 tissue homogeniser (Bertin Instruments). The cell lysate was transferred into a microcentrifuge tube. The beads were then washed with 400 μL of 10% TCA, and this volume is pooled with the previous cell lysate. Extracts were then spun at 15000 rpm at 4°C, and the supernatant was removed by aspiration. Remaining supernatant volumes were removed by aspiration after centrifugation for 1 min. Precipitated proteins were resuspended in 40 μL of buffer (100 mM Tris, pH 11, 3% SDS) supplemented with 1 $\times$ protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonyl fluoride (Sigma Aldrich), heated at 95°C for 10 min, and spun at 15000 rpm for 10 min. Protein extracts were then added with an appropriate volume of 4 $\times$ Laemmli sample buffer (200 mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 0.4% bromophenol blue), and heated for an additional 10 min at 95°C.

Proteins were typically separated on SDS-acrylamide gels and transferred onto nitrocellulose membranes. This was followed by membrane incubation with blocking buffer (LI-COR Biosciences) for 1 h at RT, before another hour of incubation with primary antibodies diluted as necessary in phosphate buffered saline (pH 7.4) with 0.1% Tween 20 (PBST). Excess antibodies were washed thrice with PBST, followed by incubation with secondary antibodies for 1 h. Membranes were washed three more times before visualization of protein bands using the Odyssey CLx Imaging system (LI-COR Biosciences).

2.5 β -galactosidase reporter assays

Yeast strains were transformed with pRS314- $P_{UPRE-CYC1}$ -*LacZ* and pCM64- $P_{HSE-SSA3}$ -*LacZ* reporter plasmids for measurement of the UPR and HSR activation, respectively [181, 188]. Strains transformed with pCM64- $P_{mHSE-SSA3}$ -*LacZ* that bears key mutations that render the promoter element unrecognizable by Hsf1 are used as negative controls for the activation of the HSR.

In brief, cells were grown to an $OD_{600} < 0.2$ /ml at 30°C [158]. Relevant strains were subjected to stress conditions for one hour by either exposure to tunicamycin at a final concentration of 2.5 μ g/ml or a temperature shift to 37°C, for the induction of the UPR and HSR, respectively. Four OD units of cells were pelleted, washed and resuspended in 75 μ L of z-buffer (60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, 1 mM $MgSO_4$, 50 mM β -mercaptoethanol). Twenty-five microliters was used to determine cell density at $\lambda=600$ nm. The remaining cell suspension was added with 50 μ L of $CHCl_3$ and 20 μ L of 0.1% SDS, vortexed vigorously for 20 s, and equilibrated at 30°C for 10 min. Seven hundred microliters of 2 mg/ml α -nitrophenyl- β -galactopyranoside (Sigma) dissolved in z-buffer was added and incubated with the mixture for a maximum of 10 min. The enzymatic reaction was quenched with 500 μ L of Na_2CO_3 , taking into consideration the reaction duration (t in min). Absorbance readings at $\lambda=420$ nm and 550 nm were taken, and corresponding β -galactosidase units were calculated $[1000 \times (A_{420} - 1.75 \times A_{550}) / (t \times 2 \times A_{600})]$.

2.6 Yeast LD visualization, live cell imaging and indirect immunofluorescence

Live cell samples were prepared by incubating 500 μ L of liquid culture onto viewing chambers pre-coated with concanavalin A (Sigma) for 10 min. Yeast LDs were stained by incubating cells with 0.05 μ g/ml BODIPY 493/503 (Invitrogen) in phosphate buffered saline (pH 7.4) for 10 min at RT, washed and resuspended in liquid medium before transferring into chambers for viewing.

For indirect immunofluorescence, log phase cells (0.3-0.8 OD₆₀₀/ml) were fixed with formaldehyde to a final concentration of 3.7% and incubated at 30°C for 90 min. Cells were gently pelleted at 1000 $\times$ g for 5 min and washed with cold 0.1 M potassium phosphate buffer (pH 7.5). Cells were then washed and resuspended in 90 μ L of 0.1 M potassium phosphate buffer (pH 7.5) with 1.2 M sorbitol before spheroplast preparation by addition of Zymolyase 20T (US Biological) at a final concentration of 1 mg/ml and further incubation at RT for 20 min. Spheroplasts were pelleted, washed and resuspended in 30 μ L of 0.1 M potassium phosphate buffer with 1.2 M sorbitol. The spheroplast suspension was coated onto wells of glass slides for 10 min at RT, followed by dehydration and fixation by methanol and acetone. After washing with Tris-buffered saline (pH 7.4), fixed cells were blocked with 5% BSA in TBST (Tris-buffered saline with 0.05% Tween 20). Appropriate primary antibodies were added (1:1000 α -Kar2, 1:1000 α -LexA or 1:200 α -GFP diluted with 1% BSA in TBST) and incubated for 90 min. After washing with TBS, cells were incubated with the secondary antibody (1:500 α -mouse IgG-DyLight 488, 1:2000 α -rabbit IgG-DyLight 550 or 1:2000 α -mouse IgG Alexa Fluor 488 diluted with 1% BSA in TBST) for 90 min and subsequently washed with TBS. Mounting medium was added before sealing of the glass slide.

Yeast cell photomicrographs were obtained using Leica DMI8 system (HCX PL APO 100x/1.4-0.70 oil immersion objective) under the control of Metamorph Ver. 7.8.10.0, or Zeiss LSM710 Microscope (100 $\times$ 1.4 NA Plan-Apochromat oil-immersion objective) under the control of Zen (Carl Zeiss MicroImaging).

2.7 Genetic screen for the temperature sensitive *scs3-1* allele

A mutant library of the *SCS3* open reading frame flanked by 500 base pairs of its endogenous promoter and 300 base pairs of its terminator (*scs3**) was generated by low-fidelity PCR through the addition of MnCl_2 to the amplification reaction to a final concentration of 0.1 and 0.05 mM. PCR amplicons were then digested with *EcoRI* and *XbaI* before being cloned into the pRS316 vector with a *URA3* selection marker. A reporter yeast strain for the screening of *scs3-1* allele was generated by replacing *SCS3* with the *KanMX* cassette in a *ire1Δ* strain in the PER genetic background bearing pRS315-*IRE1-ADE3*, which has a *LEU2* selection marker.

The pRS316-*scs3** library was transformed into the reporter strain using recombinant methods described earlier and plated on SC-URA with limited adenine (1 mg/l). Transformation plates were incubated at a permissive temperature of 25°C until colonies were observed. White or the white portion of red/white sectoring colonies (indicative of the loss of pRS315-*IRE1-ADE3*) were selected as candidates bearing plasmids that encode for temperature-sensitive mutant alleles of *scs3* (pRS316-*scs3^{ts}*) and were streaked onto two separate SC-URA plates with limited adenine, one of which was incubated at 25°C (permissive temperature) and the other at 37°C (restrictive temperature). Positive pRS316-*scs3^{ts}* candidates exhibited white or white/red sectoring at 25°C, and absent or having red colonies at 37°C. Plasmids were then extracted from these yeast strains and analyzed for key mutations in the coding sequence. Lastly, candidate plasmids were retransformed back into the PER strain for verification and comparison of the temperature-sensitive phenotype.

2.8 Total lipid extraction from whole yeast cells and microsomes

Yeast strains were grown to an OD_{600} of 0.8/ml at RT and shifted to 37°C for 2 h when necessary. For whole cell lipid extraction, 10 OD units of cells was pelleted and washed with sterile water in glass vials before being frozen at -80°C for 1 h and lyophilised under vacuum and temperature below 100 Torrs and -50°C, respectively. For microsomal fractions, 30 OD units of cells was pelleted and resuspended in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, PIC, pH 7.5) and lysed mechanically by addition of silica beads and

vortexing at 30 s intervals for 15 times. The lysate was then clarified by spinning at 800 ×g for 5 min. The microsomal fraction was then pelleted from the resultant supernatant by ultracentrifugation at 100,000 ×g for 1 h using Beckman Optima MAX ultracentrifuge with TLA-55 rotor. The pellet was resuspended in 100 µL of sterile water and homogenised by sonication for 30 min before BCA protein quantitation using BSA as a standard. A volume corresponding to 0.3 mg of total protein [193] was transferred into glass vials and lyophilised as described above.

After lyophilization, the dry weight of samples was recorded. For lipid extraction from whole cells, samples were resuspended in 100 µL of cold water and homogenised by vortexing. Afterwards, 300 µL of silica beads and 900 µL of chloroform (CHCl₃):methanol (2:1) were added before agitation for 2 h at 4°C. From here, 300 µL each of CHCl₃ and cold water were added to the mixture and vortexed for 15 s twice. The vials were centrifuged at 4250 ×g for 6 min at 4°C, and the lower organic phase was transferred to a new glass vial. The extraction step was repeated by the addition of 500 µL of CHCl₃ and further agitation for 2 h at 4°C. The two extracts were combined and dried under N₂ stream and stored in -80°C. Lipid extraction from microsomes was done similar with scaled-down reagent volumes.

2.9 Thin layer chromatography

Dried lipid extracts were reconstituted in 50 µL of CHCl₃:methanol mixed at a 2:1 ratio (20 µL for microsomal extracts), and spotted on well-dried silica thin layer chromatography (TLC) plates (Merck Millipore) using Linomat 5 (Camag). To separate TG from other lipid species, chromatograms were developed using toluene:chloroform:methanol (85:15:5) as the solvent system. Plates were dried before application of primuline solution (0.05 mg/ml in acetone:water, 80:20). Spots were visualised under UV light, and the intensity was quantified using ImageJ.

2.10 Fatty acid methyl ester (FAME) derivatization and gas chromatography with flame ionization detector (GC-FID) analysis

Separated lipids of interest were scraped from silica TLC plates, transferred into glass vials and added with 100 μ L of 1 mM pentadecanoic acid C15:0 (Sigma) internal standard and 300 μ L of 1.25 M hydrogen chloride-methanol solution (Sigma-Aldrich). Derivatization of FAMES was carried out by incubating the vials at 80°C for 1 h before cooling them down to RT and addition with 1 ml of hexane. Samples were vortexed for 30 s and centrifuged at 2000 $\times$ g for 2 min. The upper non-polar layer was transferred to a new vial using a glass Pasteur pipet. The hexane extraction step was repeated three times and the extracts were combined and dried under N₂ stream. The lipid sample was then concentrated in 20 μ L of hexane before GC-FID analysis using GC-2014 Gas Chromatograph fitted with the AOC-20i auto-injector (Shimadzu). FAME identification and quantification was carried out based on retention times relative to a standard mix of 37 FAMES (Supelco) and normalization of peak areas relative to that of the C15:0 internal standard.

2.11 Membrane yeast two-hybrid screen for ScFIT protein interactors

The bait plasmid for the expression of Scs3 and Yft2 fused with the C_{ub}-LexA-VP16 reporter tag at either N- or C-terminus under the control of a TEF1 promoter was generated by cloning the respective coding sequences into the pTLB-1 or pTMBV α plasmid [190]. Expression of the ScFIT bait proteins in the NMY51 reporter strain was verified with immunoblotting using α -LexA antibodies (1:5000 dilution) and α -rabbit IgG IRDye 680 (1:15000 dilution). Following verification of bait construct expression in the NMY51 reporter strain, the functionality of the reporter system was validated by co-expression of the bait construct with either a positive or negative control prey protein. Growth media for bait constructs that activate the reporter with the negative control prey protein was adjusted for stringency using the His3 competitor 3'-amino-1,2,4,-triazole (3'-AT). The final concentrations of 3'-AT supplemented to the growth medium that yielded minimal self-activation were 50 mM and 10 mM 3'-AT for N-terminally tagged Scs3 and Yft2, respectively. The reporter strains bearing the bait constructs were then transformed with the N_{ub}G-X prey cDNA plasmid library (DualSystems), plated and incubated at 30°C until colonies appeared. Colonies were randomly selected and plated on selective

plates supplemented with 5-bromo-4-chloro-3-indoyl- β -D-galactopyranoside (X-Gal, GoldBio) for each screen. Plasmids from blue-colored colonies indicative of positive bait-prey interaction were recovered and amplified in DH5 α competent bacteria cells. This was followed by plasmid extraction and sequencing. To reduce false-positive interactors, prey constructs were screened once more for specific interaction by retransformation back into yeast strains bearing the original bait protein. This was done in comparison to a yeast strain expressing an unrelated negative control bait construct encoding for the human CD4 T-cell surface glycoprotein. Only prey constructs that exclusively activate the reporter with the bait construct of interest are included in the final list of interactors.

2.12 Global protein ubiquitination assay

Strains expressing myc-tagged ubiquitin (Ub-myc) under the inducible *CUP1* promoter were grown to early log phase. The culture medium was supplemented with a final concentration of 100 μ M Cu₂SO₄ and incubated for 3 h for Ub-myc expression. Proteins were extracted from two OD units of cells and separated on SDS-acrylamide gels as previously described. Following transfer on nitrocellulose membranes, total protein on each lane was stained with REVERT Total Protein Stain (LI-COR Biosciences) followed by visualization on the Licor Odyssey CLx system. This was then followed by membrane blocking and incubation with antibodies against the myc epitope tag (1:1000 dilution), and α -mouse IgG IRDye 800 secondary antibodies (1:15000). Total myc-tagged protein signal was normalised against the total protein signal present as quantified through ImageStudio Lite Ver 5.2 (LI-COR Biosciences).

2.13 Cycloheximide chase assay

The cycloheximide chase assay was performed as previously described [173]. In brief, cells expressing epitope-tagged proteins of interest were grown to early log phase in appropriate selective media. Protein translation shutoff was initiated with the addition of cycloheximide (Sigma) at a final concentration of 200 μ g/ml. An aliquot of two OD units of cells were taken at the required timepoints and were added to ice-cold 10% TCA. Protein extraction and immunoblotting were done as described earlier, with antibodies against the tagged protein of

interest (α -HA at 1:2500 dilution; α -GFP at 1:1000 dilution) and Tub1 as the loading control (1:10000 dilution). Following incubation with secondary antibodies (α -mouse IgG IRDye 800, 1:15000 dilution), protein bands were visualised and quantified using ImageStudio Lite Ver 5.2 (LI-COR Biosciences).

2.14 Statistical analyses

Error bars represent the standard error of the mean (SEM) from at least three biological replicates, unless otherwise specified. All statistical analyses are carried out using two-tailed Student's paired t-test with $P < 0.05$ denoting statistical significance.

Chapter 3: Results

3.1 The ScFIT proteins, Scs3 and Yft2, are non-essential for viability under normal conditions

Sequence analyses have predicted Scs3 and Yft2 to possess transmembrane domains and are then categorised as membrane proteins [194]. In addition to this, Scs3 has been reported to localise to the cell periphery, and upon treatment with rapamycin, to the vacuole/vacuolar membrane, while the same study has reported the cytoplasmic localization of Yft2 specifically under conditions of genetic perturbation (i.e. rapamycin/hydroxyurea treatment) [195]. The lower signal intensity of fluorescently-labeled Yft2 in wild type as opposed drug-treated cells in the aforementioned study suggests that Yft2 is upregulated under these conditions. We then tagged Scs3 and Yft2 with GFP to validate their localization with reference to the ER protein marker, Kar2 (Figure 9). Our results establish the yeast FIT proteins as ER localised proteins which are present in the perinuclear, cortical and reticular networks, as similarly observed by other groups [92, 94, 97].

Figure 9. The ScFIT proteins, Scs3 and Yft2, are ER-resident proteins

C-terminal GFP fusion proteins were endogenously expressed in yeast strains by tagging the genomic loci for *SCS3* and *YFT2* with the *GFP(S65T)-HIS3MX* cassette. Co-localization of the ScFIT proteins with the ER protein marker Kar2 was shown with immunofluorescence. Scale bar, 2 μ m. Images shown are representative of three independent experiments.

3.2 LD biogenesis does not require Scs3 and Yft2

We moved on to characterise the growth phenotype of *scs3* and/or *yft2* null mutants and found that neither is essential for viability under normal conditions in minimal synthetic complete medium (Figure 10A). Expression of murine FIT proteins induced the formation of LDs in embryonic kidney cells, while shRNA-mediated knockdown of *FIT2* markedly reduced LDs in differentiated adipocytes [87]. We then investigated the role of the ScFIT proteins in LD biogenesis with reference to a LD Δ mutant strain devoid of NL biosynthetic enzymes and therefore incapable of forming LDs (*dga1 Δ iro1 Δ are1 Δ are2 Δ*) [107] and the *cho2 Δ* phospholipid perturbation mutant that has been reported to exhibit large cytoplasmic LDs [113]. All strains were cultured in minimal medium to exclude potential effects that may arise from the presence of exogenous lipids. Using BODIPY 493/503 as a LD-specific dye, we found that LD biogenesis is not affected in knockouts for either or both Scs3 and Yft2 (Figure 10B), validating results from previous studies [89, 94, 97]. This does not rule out the possibility that the ScFIT proteins contribute to the process, but rather suggests that certain compensatory mechanisms that confer robustness are in place.

Figure 10. ScFIT mutant strains remain viable and capable of LD formation

A) Cells were grown to saturation before spotting on minimal agar medium. Plates were incubated at 30°C until the formation of colonies. B) Log phase cells were grown in minimal media and incubated with the BODIPY 493/503 to stain for LDs. Deletion mutant strains for all NL biosynthetic enzymes (LD Δ) and *cho2 Δ* are shown as negative and positive controls for the presence of LDs, respectively. Scale bar, 2 μ m. Results and images shown are representative of three independent experiments.

To date, the mechanism of LD biogenesis remains obscure. No single protein has been identified as its master regulator and at most, some proteins have been found to influence the number or kinetics of LD formation in yeast. For example, yeast knockout strains for *PAH1*

(phosphatidate phosphatase) and *FLD1* (seipin) have been reported to reduce the rate and number of LDs synthesised *de novo*, respectively [75, 80]. This demonstrates the complexity of LD biogenesis and the need to identify the repertoire of proteins required for its progression.

3.3 Scs3 is essential for viability in the absence of UPR transducer Ire1

Apart from the possibility of different proteins performing redundant functions, the robustness of LD biogenesis may also be the result of activation of specific cellular response pathways. The UPR is a signal transduction pathway that is canonically activated from the accumulation of misfolded proteins within the ER lumen, but is also activated from cellular lipid perturbation [113, 133-135, 137], thereby highlighting the close association of the UPR and lipid metabolic pathways.

The formation of LDs has been reported to be stimulated under induction of ER stress in yeast cells [159], and the absence of gross changes to the transcriptional and protein expression profiles of the neutral lipid biosynthetic genes under this condition suggests that this increase in LD production is elicited through the activity of other proteins. In relation to this, several studies provide support for the functioning of *SCS3* not only in LD formation but also in the UPR. Synthetic genetic array (SGA) analyses revealed the synthetic lethality between *SCS3* and *IRE1*, which encodes for the sole UPR transducer in yeast cells [89]. Additionally, the mutant *scs3Δ* strain has been reported to activate the UPR [196], while *SCS3* was conversely shown to be transcriptionally upregulated upon UPR induction resulting from either protein stress or lipid imbalance [113, 137]. With this, we sought to further understand the role of *SCS3* within the UPR programme.

Knockout strains for genes that display synthetic lethality with *IRE1* (i.e. deletion mutants that require a functional UPR for viability) have been shown to constitutively activate the UPR even in the absence of stress induction [158, 182]. In these cases, the UPR, presumably through the transcriptional activation of approximately 381 downstream target genes including *SCS3* [137], serves as a general compensatory mechanism that alleviates the otherwise lethal phenotype in the absence of *IRE1*. Given this, along with data from previous studies [196], an

increase in the activation of the UPR in *scs3Δ* mutants is expected. However, no increase in basal UPR activation was observed in either the *scs3Δ* or *yft2Δ* single deletion mutant through a β -galactosidase reporter assay (Figure 11A), consistent with findings from a previous study that reported no difference in the mRNA splicing of the cognate transcription factor *HAC1* upon loss of ScFIT [89].

Figure 11. The failure of *ScFIT* mutants to mount a fully functional UPR is independent of LD formation

A) Activation of the UPR was measured by a reporter assay utilizing the expression of the LacZ enzyme under the *CYC1* promoter with the UPR element. Cells were either treated with the *N*-linked glycosylation inhibitor tunicamycin (Tm) or the carrier DMSO for 1 h prior to harvesting for the assay. Data shown is the mean $\pm$ SEM from three independent experiments. Statistical analysis was subjected to paired two-tailed Student's t-test. B) WT and *scs3Δ* mutant cells were treated with Tm for 1 h before LD staining with the fluorescent BODIPY 493/503 dye. Scale bar, 2 μm . Images shown are representative of three independent experiments.

The differences in UPR activation in these mutant strains may be accounted for by the specific assay that was utilised. As fluorescent proteins typically have long half-lives, stress-induced expression of GFP may lead to a significantly higher activation readout due to the sustained fluorescent signal [197]. While a small yet significant decrease in UPR activation was observed in the *ScFITΔ* mutant, such a change may not necessarily be sufficient to yield physiologically relevant phenotypes, especially given the broad compensatory effect of the

UPR in yeast cells [158]. Interestingly, we found that upon treatment of cells with tunicamycin (Tm) to induce ER-specific protein stress, the absence of *SCS3* in the single *scs3Δ* deletion and *ScFITΔ* double deletion mutants led to the reduction in UPR activation (~25% and ~30% reduction, respectively) in comparison to that of WT (Figure 11A).

Increased LD biogenesis has been extensively observed in cells under stress conditions. Introduction of oxidative stressors or the attenuation of antioxidant capacities of cells result in the formation of LDs [67, 198, 199]. While the extent of UPR activation and the resulting transcriptional profile differs between proteotoxic and lipid stress, a similar increase in lipogenic markers and concomitant LD formation is observed [113, 159, 160]. It remains to be determined whether LDs contribute to stress induction or if this is reflective of the adaptive role of LDs to mitigate the otherwise deleterious effects of stress. However, all these undoubtedly highlight the complex integration of LDs in stress response pathways.

In addition to this, the UPR regulates metabolic pathways to a certain extent under normal physiological conditions [200], and similarly orchestrates the complex transcriptional metabolic reprogramming under ER stress induction [113]. As lipogenic pathways constitute one of the major effectors of the UPR [201] and LDs have been associated with stress conditions, the putative function of *Scs3* in LD formation may then provide a rationale for its transcriptional upregulation in the UPR programme. With this, we further hypothesised that the inability of *scs3Δ* mutants to mount a maximal UPR under stress conditions may translate to the impairment of LD formation as part of the stress response programme. We once again made use of fluorescent LD staining in *scs3Δ* mutants challenged with Tm-induced stress to evaluate gross changes in LD formation and found that mutant cells are still able to form LDs to the same extent as that of WT cells (Figure 11B).

FIT2 deletions in mammalian cell lines have been reported to exhibit a defect in LD biogenesis together with an increase in ER stress markers [94]. Since yeast cells rely solely on the Ire1 sensor as opposed to the three UPR axes in higher eukaryotes (Figure 5), the yeast UPR intuitively coordinates a larger network of effectors, which in turn results in a more generalised

yet highly robust adaptive response. From here we sought to determine the extent by which the UPR compensates for the absence of *SCS3*, particularly in respect to LD formation. However, a *scs3Δire1Δ* mutant is synthetically lethal, thus rendering conventional deletion strategies unusable in titrating the phenotypic effects of the UPR upon the loss of *SCS3*. To address this, we employed a genetic screening strategy that takes advantage of the dependence of *scs3Δ* mutants on the presence of *IRE1* to generate a conditional temperature sensitive *scs3* allele (*scs3^{ts}*) that is functional at a permissive temperature of 25°C but not at the restrictive temperature of 37°C (Figure 12) [202].

The screen utilises an *ire1* null mutant bearing a *LEU2* selection plasmid encoding for *IRE1* and *ADE3* (pRS315-*IRE1-ADE3*) in the PER background [182]. It is important to note that the PER yeast strain background lacks two enzymes in the adenine biosynthetic pathway, namely *ADE2* and *ADE3*. *ADE2* encodes for pyrophosphate-aminoimidazole carboxylase, which when mutated leads to the initiation of adenine biosynthesis and the accumulation of the red-pigmented intermediate aminoimidazole ribotide (AIR) in the vacuole, thereby causing the growth of red colonies [203, 204]. *ADE3* encodes for C₁-5,6,7,8-tetrahydrofolate synthase [205] that lies upstream in the pathway, and an *ade3* mutation prevents the formation of AIR. Therefore, the PER genetic background gives rise to white yeast colonies, which when transformed and selected for plasmids bearing the *ADE3* gene leads to the formation of red colonies. Finally, the reporter strain for the screen was generated by subsequently replacing the genomic *SCS3* ORF with the *KanMX* cassette.

PER $\Delta ire1 \Delta scs3$

Figure 12. Schematic diagram for the screening of *scs3-1* allele

The *SCS3* ORF was replaced with the *KanMX* resistance cassette in the *ire1* null reporter strain bearing the pRS315-*IRE1-ADE3* plasmid. Red colony pigmentation is due the accumulation of aminoimidazole ribotide and is a resultant phenotype of pRS315-*IRE1-ADE3* retention. Figure adapted from [202] with permission.

To validate the use of the reporter strain, a colony sectoring assay was employed (Figure 13A). In the absence of selective pressure, centromeric plasmids that are not required for viability are randomly lost through the course of cell division [202]. While retention of pRS315-*IRE1-ADE3* leads to the red pigmentation of colonies, the loss of the same plasmid

phenotypically results in the formation of red/white sectoring colonies. The *ire1Δscs3Δ* mutant, but not the *ire1Δyft2Δ* mutant, specifically gave rise to red colonies when grown in rich media without any form of plasmid selective pressure (Figure 13A). This provides strong support for the interdependence between *IRE1* and *SCS3* and that one is required for viability in the absence of the other, consistent with SGA results showing synthetic lethality between these two genes [89].

Figure 13. Colony sectoring assay reveals essentiality of *IRE1* in the absence of *SCS3*

A) The *ire1Δscs3Δ* mutant exhibited red coloured colonies indicating the requirement for the pRS315-*IRE1*-*ADE3* plasmid for viability. B) Final candidates for the temperature sensitive *scs3* allele in the PER yeast genetic background were evaluated for robust growth phenotype at the permissive temperature of 25°C. WT yeast cells with a W303 genetic background was used as a control. C) Strains in the PER genetic background were grown at 25°C in minimal media from which dilutions were prepared and spotted onto plates. Plates were incubated at the specified temperatures until colonies were observed. Results shown are representative of three independent experiments.

The validated reporter strain was transformed with a mutant *scs3* ORF library cloned into a *URA3*-based plasmid (pRS316-*scs3*) and grown in SC-Ura plates with limited adenine at 25°C until the colony colour was evident. A total of 208 colonies exhibiting white or red/white sectoring phenotype were similarly restreaked onto two plates, one incubated at a permissive

temperature of 25°C and the other at the restrictive temperature of 37°C. From this number, 31 yeast colonies harbouring candidate clones for the temperature sensitive *scs3* (pRS316-*scs3^{ts}*) were identified. These grew as white or red/white sectoring colonies at 25°C, while having no growth or red colonies at 37°C. Eight final candidates were chosen on the criterion of robust growth at 25°C and arbitrarily named *scs3-1* to *scs3-8* (Figure 13B). Finally, the strain designated as *scs3-1* was identified as having the most pronounced temperature-sensitive phenotype among all the clones and was used for succeeding experiments (Figure 13C).

From here, the plasmid bearing the *scs3-1* allele was recovered from yeast, amplified in *E. coli*, and subjected to sequence analysis to identify the mutations within the ORF. Two amino acid mutations were identified in the *scs3-1* allele, D277G and I328V. Based on topological prediction, these fall within the cytosolic loop linking transmembrane domain IV and V and within transmembrane domain V itself, respectively (Figure 4).

3.4 Triacylglycerols accumulate within the ER in the absence of both Scs3 and Ire1

It has been recently reported that in *scs3Δ* and/or *yft2Δ* mutants, LDs remain irreversibly tethered to the ER and are wrapped by a membrane suggested to be continuous to that of the ER [92]. We hypothesised that while these knockout strains are fully capable of forming LDs (Figure 10B, 11B), it is possible that these LDs remain tightly associated with the ER, and this phenotype could not readily be detected with fluorescent labelling alone. In addition, we considered the potential influence of the UPR in this process and investigated further utilizing the conditional *scs3-1* mutant in *ire1* null cells (*ire1Δscs3-1*).

Strains were cultured in minimal media at 25°C followed by a shift to the restrictive temperature of 37°C and further incubation for two hours. Microsomes, which correspond to the ER fraction, were then isolated from harvested cells, and total lipids were extracted from the resulting fraction. TG, one of the two main neutral lipid species in LDs, were separated from the total lipid pool by thin layer chromatography (TLC) (Figure 14A). From here we found an increase in the amount of TG present within the microsomal fraction in the *ire1Δscs3-1*

strain (Figure 14B-C). This may be due to the failure of LDs to bud off from the ER as reported previously [92]. However, such an increase was not evident in the *scs3-1* strain alone, thereby suggesting that the presence of the UPR may exert a suppressive effect for this phenotype.

Figure 14. Microsomal TG increases in the absence of both SCS3 and the UPR

Strains were grown to log phase at 25°C in minimal media, after which the temperature was shifted to 37°C for 2 h. A) Extracted microsomal lipids were separated on TLC plates. TG, triacylglycerol; DG diacylglycerol. B) Changes in TG acyl chain profiles. All FA species were normalised against WT levels. ns, not significant. C) Relative TG abundance was determined by FAME analysis through GC-FID and normalised against WT. Data shown are the mean ± SEM from three independent experiments. Statistical analysis was subjected to paired two-tailed Student's t-test.

3.5 Cells exhibit ER membrane clustering in the absence of Scs3 and the UPR

To confirm that the observed retention of TG in the microsomes (Figure 14) resulted from the failure of LDs to physically separate from the ER membrane, transmission electron microscopy was performed on *ire1Δscs3-1* cells in collaboration with the research group of Dr. William Prinz (NIH). When grown under the permissive temperature of 25°C, LDs are bound by ER membranes (Figure 15A-B). This closely resembles the phenotype observed in *scs3Δ* cells upon induction of LD biogenesis [92] and could be the result of the partial inactivation of the mutant Scs3 protein encoded by *scs3-1* at 25°C, consistent with the slight growth defect observed in *ire1Δscs3-1* compared to WT cells at that temperature (Figure 13C). This suggests that although a partially inactive Scs3 is sufficient to rescue its synthetic lethality with *IRE1* loss, its prolonged effects could result in a phenotype like that of a complete SCS3 deletion mutant.

Figure 15. LDs are enveloped in ER-derived membranes in the absence of Scs3 that is exacerbated upon loss the UPR

A-B) The *ire1Δscs3-1* strain was grown to log phase at 25°C in minimal media before preparation for imaging. C-D) Log phase *ire1Δscs3-1* cell cultures were shifted to 37°C for 2 h before imaging. Scale bars for A and C, 0.4 μm. Scale bars for B and D, 200 nm. LDs are highlighted in yellow and ER membranes are indicated by red arrows. Cell sectioning and transmission electron photomicrographs were prepared by Dr. Vineet Choudhary in collaboration with Dr. William Prinz's research group (NIH).

Interestingly, complete inactivation of Scs3 functionality in tandem with *IRE1* deletion following a temperature shift to 37°C exacerbates this aberrant LD-associated membrane structures, as majority of cells display LDs that were extensively entangled in electron-dense structures that include the ER (Figure 15C-D). Under stress conditions, the UPR orchestrates a cascade of cell signalling events that aim to restore cellular homeostasis. UPR-dependent modulation of

phospholipid biosynthesis for membrane expansion has been shown to be particularly critical in coping with stress [206], thereby emphasizing the importance of maintaining proper membrane dynamics. Our results suggest that upon loss of both *Scs3* and the buffering capacity of the UPR, the integrity of the ER membrane is severely compromised, and this in turn not only influences the proper maturation of nascent LDs but also cell viability (Figure 15, Figure 13C).

It has been recently reported that deletion of *SCS3* resulted in ER membrane clustering but had no effect on *de novo* LD formation [94], thereby supporting our own findings (Figure 10, Figure 15). Similar ER membrane aberrations in *FIT2* mutant mammalian cell lines were observed in the same study. This strongly alludes to the alternative hypothesis that *ScFIT* could be but one of several interconnected proteins required for the LD biogenesis and that *ScFIT* proteins may serve a different function altogether.

Several studies have reported on the involvement of *ScFIT*, particularly *SCS3*, in phospholipid metabolism. *SCS3* was first characterised as an essential gene to produce inositol [96], a crucial intermediate for the synthesis of phospholipids. Moreover, *ScFIT* mutants were found to have genetic interactions with enzymes involved in the synthesis of PE and PC [89, 207], the predominant membrane phospholipid species [20]. Mutants of genes encoding for these phospholipid biosynthetic enzymes, such as *OPI3* and *ICE2*, are characterised by alterations in ER membrane morphology and gross changes in LD levels [85, 113]. In the same way, a severe genetic interaction has been reported for the concurrent deletion of *SCS3* and *SEY1*, one of the key players in ER membrane dynamics [89, 208].

Consistent with this, the mammalian *FIT2* has been reported to exhibit lipid phosphatase activity *in vitro*, with the membrane phospholipid intermediates phosphatidic acid and lysophosphatidic acid as substrates. In addition, this enzymatic function has been hypothesised to be essential in the maintenance of ER membrane homeostasis, as catalytically inactive mutants of either *FIT2* or *Scs3* fail to rescue the ER clustering phenotype [94]. This strongly supports the contention that *Scs3* may primarily be involved in membrane

phospholipid homeostasis and that the heretofore effects on LD formation may be a secondary effect that follows insults to ER membrane integrity.

3.6 Scs3 interacts with components of the proteostatic machinery

To date, the exact function of the ScFIT proteins has remained enigmatic, and despite considerable efforts to ascertain the molecular mechanism by which these proteins exert their functions within the cell, a definitive consensus has yet to be reached. The initial paradigm of the FIT proteins being direct LD-forming proteins through its lipid binding capacity [88, 91] is shifting towards an alternative hypothesis of their broader involvement in the maintenance of membrane homeostasis [92, 94].

Moreover, information on the interactors of ScFIT has been limited to the genetic level [89], and the identification of physical interactors has been minimal thus far [209, 210]. To gain further insight into the physiological relevance of ScFIT proteins within the cell, we employed the membrane yeast two-hybrid (MYTH) screen to identify their potential interacting partners [190]. This screen utilises two stable moieties of the yeast ubiquitin molecule, namely its C-terminal half that is fused to an artificial transcription factor (C_{ub} -LexA-VP16) and its N-terminal (N_{ub}) half, which are then tagged to the protein of interest (bait) and a library of yeast proteins (prey library), respectively. Interaction between the bait and a prey protein leads to the reconstitution of the ubiquitin molecule. Subsequent recognition and cleavage of ubiquitin by the cellular machinery liberates the transcription factor, which then induces the expression of reporter genes within the nucleus.

The topology of the ScFIT proteins having both N- and C-termini facing the cytosol makes them ideal for this screen as the (C_{ub} -LexA-VP16) epitope could be placed on either end to obtain a more comprehensive list of potential interacting partners. To this end, the coding sequence of each ScFIT protein was cloned into two MYTH bait plasmids, pTMBV α and pTLB1, which placed its expression under the control of a strong *TEF1* promoter. The screen details are summarised in Table 4.

Table 4. Summary of the MYTH screens for ScFIT interactors

Bait construct	Colonies screened	Putative interactors	Specific interactors	Unique interactors
(LexA-VP16-C _{ub})-Scs3	480	159	35	31
Scs3-(C _{ub} -LexA-VP16)	288	158	66	39
(LexA-VP16-C _{ub})-Yft2	288	123	33	3
Yft2-(C _{ub} -LexA-VP16)	288	224	55	15

Bait constructs were validated, and screening conditions for each were optimised with 3'-amino-1,2,4,-triazole (3'-AT) supplementation in the selection media to reduce the occurrence of false positives. Following this, 1344 colonies were collectively screened for all reporter strains. From these, 664 colonies were positive for bait-prey interaction as manifested by blue colony growth on X-Gal supplemented selective media, and are designated as putative interactors (Figure 16). These were further validated for specificity towards the bait protein of interest in comparison to the single-pass human cluster of differentiation 4 (CD4) receptor protein, which served as a negative control. From these, 189 showed specific interactions with the ScFIT proteins. Following sequence analysis, 88 unique protein interactors were identified. Considering the overlap in protein interactors, the MYTH screen identified a total of 73 bona fide and unique interactors for the ScFIT proteins, which were further categorised according to cellular function (Figure 17, Appendix Table S1). Moreover, our screen results show that more than half of the identified Yft2 protein interactors is shared with that of Scs3, thereby supporting a certain degree of functional redundancy between the two (Figure 18).

Surprisingly, only few of the ScFIT protein interactors identified with the screen are directly involved in lipid metabolism, suggesting that ScFIT proteins may not function extensively in that cellular process. It could also be noted that while the encoded proteins of genes that *ScFIT* had high degrees of genetic interactions with, such as *ICE2*, *SEY1* and *UBX2* [89, 207], were not identified with the MYTH screen, this does not exclude the presence of a physical interaction. However, this alternatively suggests that they function in parallel but independently in the same cellular process, and that the loss of both is detrimental to cell viability.

Figure 16. Validation of ScFIT protein interactor specificity

Prey plasmids encoding for the putative interactors of the ScFIT proteins were evaluated for specificity by transformation into yeast reporter strains bearing the A) pTLB-SCS3, B) pTMBV α -SCS3, C) pTLB-YFT2, and D) pTMBV α -YFT2. Colony growth and blue pigmentation resulting from the positive interaction between the bait and prey constructs was compared to a negative control yeast strain artificially expressing the human CD4 membrane receptor tagged with the LexA-VP16-C_{ub} reporter moiety.

Figure 17. The ScFIT interactome as identified by membrane yeast two-hybrid screening
Physical protein interactors of C- and N- terminal reporter-tagged ScFIT proteins are grouped according to function.

Interestingly, several proteins that function in proteostasis and the UPS have been found to interact with Scs3 (Figure 17, 18). Of note are the J-protein chaperone Zuo1 and the Hsp70 chaperone Ssb2, both of which have been reported to function in protein quality control [211-213]. Doa10 is one of the key E3 ubiquitin ligases in yeast, and is involved in ER-associated degradation (ERAD) of proteins [192, 214, 215]. Lastly, Pup1 is a subunit of the yeast 20S proteasome core [216, 217]. Taken together, these suggest that Scs3 may function to a certain extent in protein quality control pathways, specifically in the UPS.

Figure 18. ScFIT interactor frequency

The frequency by which each unique protein interactor was identified in ScFIT reporter constructs tagged in either N or C- terminus is represented in a heat map. Proteins are categorised according to function as in Figure 14, A) vacuolar function, B) endomembrane transport, C) transmembrane transport, D) protein processing, E) lipid metabolism, F) cell wall maintenance, G) small molecule metabolism, H) cytoplasmic translation, I) unknown functions and J) other functions.

3.7 ScFIT mutants are sensitive to proteotoxic stress

As membrane homeostasis is crucial in the maintenance of proper ER functions, the defects in the ER membrane exhibited by *scs3Δ* mutants (Figure 15) [92, 94] may lead to the impairment

of not only LD maturation, but also other ER-related processes. One such process is ERAD, a component of the proteostatic UPS. Proteins that fail to fold correctly in the ER are retained and targeted towards ERAD, the terminal phase of ER protein quality control. The accumulation of misfolded proteins upon saturation of the ERAD pathway activates the UPR, which then compensates for the protein load by transcriptional upregulation of the ERAD machinery components [113, 218].

In connection to this, an aberrant ER membrane morphology has been previously reported for yeast *ubx2Δ* mutants, characterised by clustered ER membrane whorls [208]. Apart from its role in maintaining normal membrane lipid saturation through the transcriptional modulation of the *OLE1* desaturase, Ubx2/Sel1 is an ER-membrane protein that directly participates in ERAD, wherein it interacts with and bridges the Cdc48 ATPase with the main yeast E3 ubiquitin ligases, Doa10 and Hrd1, for protein substrate degradation [208, 219, 220]. Ubx2 has additionally been implicated in LD biogenesis [86], and has been shown to have a severely negative genetic interaction with *scs3Δ* and *ScFITΔ* mutants [89]. With this, the possibility that these proteins may synergistically function in membrane homeostasis and protein quality control and turnover warrants further investigation.

With the association of *SCS3* with the UPR, we then investigated how the loss of *ScFIT* would affect the capacity of cells to cope with protein stress. First, we pharmacologically induced ER-specific proteotoxicity to cells by growing them in the presence of the *N*-linked glycosylation inhibitor tunicamycin. While the mutant strains remained viable under such proteotoxic stress condition, *scs3Δ* cells exhibited a growth sensitivity that is further exacerbated in *ScFITΔ* (Figure 19), in agreement with previous reports [89]. The same induction of protein stress by the reducing agent dithiothreitol has been reported to negatively impact the growth of *ubx2Δ* mutants [221]. This observation not only supports the physiological relevance between the function of *Scs3* with the UPR (Figure 11A, 13), but also suggests that the absence of *ScFIT* may impair the proteostatic network.

Figure 19. *ScFIT* Δ mutants exhibit growth sensitivity to protein stress

ScFIT mutant strains were grown in selective medium prior to dilution and spotting on vehicle or Tm-supplemented media. Culture plates were incubated at 30°C until colonies are observed. Results shown are representative of three independent experiments.

3.8 The clearance of ERAD client proteins is impaired in *ScFIT* mutants

As the accumulation of misfolded proteins and the ensuing proteotoxicity is closely related to the ability of cells to efficiently process client proteins, we hypothesised that the sensitivity to tunicamycin exhibited by *scs3* Δ and *ScFIT* Δ mutants may be the result of impaired protein degradation pathways, as in the case of *ubx2* Δ mutants having impaired turnover of both misfolded ER and cytosolic substrates [222]. Following the identification of UPS machinery components as protein interactors of *Scs3*, we then investigated the turnover of known ERAD substrates in mutants of *ScFIT* through the cycloheximide chase assay. We first utilised native proteins as substrates to determine protein turnover in *ScFIT* Δ mutants. To this end, we expressed HA-tagged constructs of *Sbh2*, *Yeh1*, *Pgc1*, all of which are dependent on Doa10-mediated ERAD for its normal degradation [47, 192] (Figure 20).

Figure 20. *ScFITΔ* mutants can degrade natively folded proteins

Protein levels of the known Doa10-dependent ERAD substrates, A) HA-Sbh2 and B) Yeh1-3×HA, and C) 3×HA-Pgc1, were monitored at the indicated timepoints following the attenuation of protein translation with cycloheximide. Chase experiments for B and C were done with cells grown in media supplemented with 0.1% oleic acid. Data shown are the mean $\pm$ SEM from three independent experiments. Statistical analysis was subjected to paired two-tailed Student's t-test.

Our results indicated that the rates of Sbh2 and Yeh1 turnover in *ScFITΔ* mutants does not differ from that of WT cells (Figure 20A-B). Interestingly, the degradation of Pgc1 was

significantly accelerated in *ScFITΔ* mutants (Figure 20C). Along with the identification of Pgc1 as a Doa10-dependent ERAD substrate, its proper localization dynamics between the ER and LD membranes was found to be critical in determining its stability [47]. Doa10 reportedly recognises ER-localised Pgc1 through its hairpin loop, which then serves as a degron that concentrates Pgc1 on the surface of LDs. As LDs fail to properly mature and remained tethered to ER membrane in the absence of the ScFIT proteins [92] (Figure 15), the lateral diffusion of the pool of Pgc1 proteins to the ER may be increased in *ScFITΔ* mutants, resulting in continual degradation by Doa10 [32]. We hypothesised that native proteins in their proper conformation may not illicit a proteotoxic effect on *ScFITΔ* cells, and that an otherwise compromised protein degradation pathway in this mutant could remain fully capable of clearing these endogenous proteins. Following protein translation shutoff, we monitored the protein levels of epitope-tagged versions of the classical misfolded Prc1/carboxypeptidase Y mutant (CPY*-HA) and an engineered misfolded variant of the Pep4 vacuolar protease (ngPrAΔ295-331-HA), both of which has been shown to be degraded through the activity of the Hrd1 E3 ligase [158, 185] (Figure 21).

Figure 21. Degradation of Hrd1-dependent ERAD substrates is impaired in *ScFITΔ* cells Protein levels of the model Hrd1-dependent ERAD substrates, A) CPY*-HA and B) ngPrA*Δ295-331-HA, were monitored at 0, 30, and 60 min timepoints following the attenuation of protein translation with cycloheximide. Data shown are the mean $\pm$ SEM from three independent experiments. Statistical analysis was subjected to paired two-tailed Student's t-test.

Interestingly, we have identified that upon loss of both ScFIT proteins, mutants are unable to efficiently degrade these ERAD client proteins, as shown by the relative stabilization of CPY*-HA and ngPrA Δ 295-331-HA in *ScFIT* Δ cells compared to its WT counterpart at the end of the 60 min chase duration. It could be noted that neither of the single deletion mutants (*scs3* Δ or *yft2* Δ) resulted in a stabilization of the ERAD substrates. As *YFT2* is reported to have been the result of the segmental duplication of *SCS3*, the two may share a redundant yet hitherto poorly understood function [89]. This is supported by the more pronounced growth sensitivity to tunicamycin in the double *ScFIT* Δ mutant in comparison to a mild defect in *scs3* Δ cells (Figure 19). This, along with the broader range of Scs3 protein interactors (Figure 17), also suggests of an asymmetric redundancy wherein *YFT2* only partially compensates for the absence of *SCS3* functionality in the ERAD pathway, which ultimately results in less apparent phenotypic defects in *yft2* Δ mutants (Figure 19, Figure 21).

The expression of the misfolded substrate CPY*-HA has additionally been shown to strongly activate the UPR, as does the disruption of ERAD function [137, 223]. Given the sensitivity of *ScFIT* Δ mutants to proteotoxic agents (Figure 19) [89], the expression of terminally misfolded protein substrates (Figure 21), as opposed to native proteins (Figure 20), may already be a form of stress *per se* that overwhelms the UPS machinery, thereby giving rise to the defect in ERAD.

3.9 Protein turnover defects in *ScFIT* Δ mutants is accompanied by impaired protein ubiquitination

The ERAD pathway is a step-wise process that enjoins several components of the proteostatic machinery to mediate substrate recognition, polyubiquitination, cytosolic retrotranslocation and finally degradation (Figure 7) [167]. Given this, we hypothesised that the aberrant accumulation of misfolded substrates in *ScFIT* Δ mutants may be potentiated by the failure to effectively mark them with the appropriate degradation signal. To investigate this, we overexpressed myc-tagged ubiquitin (Ub-Myc) through and inducible *CUP1* promoter and quantified the extent of total protein ubiquitination in cells by immunoblotting. In agreement with our hypothesis, we observed that the amount of ubiquitinated proteins in *ScFIT* Δ mutants

was significantly reduced by approximately 38% relative to that of WT cells, with a less pronounced reduction of 18% in *scs3Δ* single mutants (Figure 22).

Figure 22. Global protein ubiquitination is reduced in *ScFITΔ* mutant cells

Cells were grown in minimal media to early log phase followed by extrachromosomal expression of myc-tagged ubiquitin with 100 mM Cu_2SO_4 into the culture medium. Cell lysates were ran on SDS-PAGE, and total Ub-Myc signal was normalised against the corresponding total protein levels (TCE). Data shown are the mean $\pm$ SEM from three independent experiments. Statistical analysis was subjected to paired two-tailed Student's t-test.

Since the stabilization of protein substrates was accompanied by a decrease, rather than an increase in high molecular weight ubiquitin antibody-reactive proteins, the inefficient turnover of the said substrates is likely due to a failure to mark them correctly for degradation and not because of efficient clearance in the proteasome. Our research group has recently reported that ER transmembrane proteins are unexpectedly destabilised upon perturbation of the ER membrane bilayer despite being transcriptionally upregulated [184]. This highlights the importance of maintaining membrane homeostasis to preserve the integrity of proteins crucial for ER functioning. In ERAD, polyubiquitination constitutes a critical component as it provides the signal that bridges the process of substrate selection and translocation, and while there are evidences of ubiquitination-independent degradation pathways, majority of proteins still rely on ubiquitin as a signal for clearance. Interestingly, the ubiquitination factor Cue1 has been identified as one of the destabilised proteins under membrane bilayer disruption [184]. The Cue1 protein recruits another ERAD factor Ubc7 and binds ubiquitin chains to allow its

subsequent linkage to substrates by either Hrd1 or Doa10 [224, 225]. It may then be possible that the altered ER membrane morphology in *ScFIT* mutants may similarly affect the stability, if not the functioning, of Cue1 or other membrane-bound ubiquitination factors in the ERAD complex (Figure 7).

3.9 Protein turnover defect in *ScFITΔ* mutants is not confined to the ER

The UPS does not exclusively accommodate client substrates that emerge from ERAD pathways. Protein quality control in the cytoplasm (cytoQC) canonically recruits a set of ubiquitination factors unique from that of ERAD, but ultimately culminates in the clearance of client proteins via the 26S proteasome. Two main E3 ligases mediate cytoQC, the nuclear San1 and the cytosolic/nuclear Ubr1 (Figure 8), each with its distinct set of cofactors and client proteins [172, 174, 175]. Interestingly, there have been several reports of crosstalk between ERAD and cytoQC. The Ubr1 ubiquitin ligase, while primarily responsible for the ubiquitination and clearance of cytosolic misfolded proteins, was found to be similarly required for the clearance of known membrane ERAD substrates [226, 227]. Meanwhile, Doa10 has also been reported to participate in the degradation of soluble cytosolic proteins [214].

Taken together with the reduction in global protein ubiquitination (Figure 22), all these present the possibility that defects in ubiquitination-dependent protein turnover may extend to misfolded proteins outside the ER. To address this question, we performed the cycloheximide chase utilizing HA-tagged constructs of two San1-dependent cytoQC substrates, namely the Pep4 vacuolar protease devoid of its signal sequence that causes its mislocalization and subsequent misfolding (Δ ssPrA-HA), and a mutant GFP with a disordered β -barrel conformation (Δ 2GFP-HA) [173, 175]. Consistent with our results using misfolded ERAD substrates (Figure 21), we found that *ScFITΔ* mutants are unable to efficiently clear away both cytosolic substrates compared to WT cells or either of the single mutants (Figure 23).

Figure 23. Cytoplasmic protein quality control is similarly impaired in *ScFIT* Δ mutants

Protein levels of the cytoplasmic misfolded protein substrates, A) $\Delta 2\text{GFP-HA}$ and B) $\Delta \text{ssPrA-HA}$, were monitored at 0, 30, and 60 min timepoints following the attenuation of protein translation with cycloheximide. Data shown are the mean $\pm$ SEM from three independent experiments. Statistical analysis was subjected to paired two-tailed Student's t-test.

A unique feature of the San1 and Ubr1-mediated protein quality control pathways is the trafficking of its client proteins into the nucleus for ubiquitination before being sent to the proteasome. This nuclear import relies on the presence of the chaperones, Ydj1 and Sse1 [173-175]. To narrow down which between the steps of nuclear import and substrate ubiquitination may have been affected and could therefore have led to the stabilization of client proteins in *ScFIT* Δ mutants, we performed indirect immunofluorescence to ascertain the localization of $\Delta 2\text{GFP-HA}$ and $\Delta \text{ssPrA-HA}$ (Figure 24). In WT cells, $\Delta 2\text{GFP-HA}$ and $\Delta \text{ssPrA-HA}$ is primarily localised in the nucleus as marked by the nuclear stain DAPI, consistent with previous reports [173, 175]. As a similar nuclear localization of both proteins was observed in *ScFIT* Δ mutants, the increased stability of these proteins was not due to the failure in trafficking them into the pertinent cellular compartment, but rather in a process that lies downstream of it.

Figure 24. The localization of misfolded cytoplasmic protein substrates is unaffected in *ScFIT Δ* mutants

The nuclear localization of A) $\Delta 2\text{GFP-HA}$ and B) $\Delta \text{ssPrA-HA}$ were determined with immunofluorescence, as indicated with yellow arrows. DAPI and Kar2 protein signals mark the nucleus and ER membrane, respectively. Scale bar, 2 μm . Images shown are representative of three independent experiments.

Taken together, our results suggest that while cytosolic chaperone activity required for the import of cytoQC substrates remains intact in *ScFIT Δ* mutants, the protein factors that are responsible for client polyubiquitination may be compromised, consistent with the marked reduction in ubiquitinated proteins in this mutant (Figure 22). However, San1 is a soluble nuclear protein, therefore its stability and activity may unlikely be directly affected by aberrations in membrane properties [184]. While the nuclear localization of the cytoQC substrates (Figure 24) has been shown to correspond to the extent of its ubiquitination [175], the possibility that their consequent delivery to and clearance in the 26S proteasome may be affected could not be excluded.

3.10 Activation of the cytosolic heat shock response is dampened in *ScFITΔ* mutants

Whereas proteotoxic stress in the ER activates the UPR, the presence of misfolded proteins in the cytosol induces the activation of the HSR [228], as does the induction of heat stress by growth at elevated temperatures [229]. Under this adaptive cellular response, molecular chaperones, ubiquitination factors and proteasomal subunits are upregulated to cope with the physiological stress and increase the rate of protein turnover [230, 231]. Given the stabilization of cytoQC substrates in *ScFITΔ* cells, we hypothesised that the HSR may not be sufficiently activated in this mutant. Through a HSE-driven β -galactosidase reporter assay, we found that HSR activation was indeed significantly reduced in *ScFITΔ* mutants compared to WT under either basal or heat stressed conditions (Figure 25). These results agree with a previous report on the dependence on an intact HSR for the prevention of proteotoxicity even under normal physiological conditions [149].

Figure 25. Heat shock response activation is dampened in *ScFITΔ* mutants

Activation of the HSR in cells grown under normal or heat stressed conditions was measured by a reporter assay utilizing the expression of the LacZ enzyme under the SSA3 promoter with the heat shock element (HSE). A similar construct with an unrecognizable HSE mutant (mHSE) was used as negative controls. Arbitrary values under the two different conditions were normalised against the corresponding WT values. Data shown are the mean $\pm$ SEM from three independent experiments. Statistical analysis was subjected to paired two-tailed Student's t-test.

The effectors of the HSR not only accommodate the increased protein folding requirement of cells, but also growth in elevated temperatures. HSR activation by attenuation of proteasome function has been reported to confer increased thermotolerance through the upregulation of molecular chaperones [232]. Surprisingly, the drastic reduction in *ScFIT* Δ mutant HSR activation did not compromise growth under heat stressed conditions, with growth comparable to that of WT cells (Figure 26). The normal growth of *ScFIT* Δ mutants under heat stress, compared to its pronounced sensitivity to proteotoxicity (Figure 19), provides strong support for the function of *ScFIT* in maintaining the integrity of the proteostatic machinery of cells, as opposed to a generalised stress response.

Figure 26. Thermotolerance is not affected in *ScFIT* Δ mutants

WT and mutant cells were grown, diluted and spotted on minimal medium before incubation under normal (30°C) or heat stress (37°C) conditions until colonies appeared. Results shown are representative of three independent experiments.

Chapter 4. Discussion

Lipid droplets have been increasingly implicated in disease pathophysiology. Despite this, our understanding of its involvement is obscure at best as LD biology is still in its infancy, and more mechanistic insight into LD formation is warranted to grasp its relevance and importance in physiological processes. From the simple budding yeast, several proteins have been identified to influence LD generation [75, 77, 80]. Among these, the FIT2 class of proteins has similarly gained much interest in recent years, but its initial putative role in LD formation as a lipid-binding protein has recently been contested in favor of a broader function in membrane homeostasis. However seemingly disparate, the identification of lipid phosphatase activity in FIT2 may not be mutually exclusive with previous reports of its involvement in LD biogenesis. Given this, the molecular mechanism by which these two processes are linked is poorly understood, as well as its potential implication for the normal functioning of cells outside the context of LD formation. In this study, we report on the involvement of the yeast FIT homologs (*ScFIT*) not only in the maintenance of ER membrane homeostasis, but also in coordinating two cellular stress response pathways, namely the UPR and HSR, and its consequent impact protein quality control.

4.1 *ScFIT* proteins maintain ER homeostasis through membrane lipid regulation

The absence of either one or both *ScFIT* genes, *SCS3* and *YFT2*, did not yield defects in growth and LD forming capacity of mutant cells (Figure 10), in agreement with previous studies [89, 94]. While *ScFIT* mutants did not exhibit a constitutively activated UPR, we found that loss of *SCS3* impaired maximal UPR activation under stress conditions (Figure 11A), and that *SCS3* is specifically required for viability in the absence of the sole yeast UPR transducer, *IRE1* (Figure 13C). The conditional knockout of *SCS3* in conjunction with *IRE1* deletion led to a significant increase in the neutral lipid TG within the ER (Figure 14), along with exacerbated defects in ER membrane morphology (Figure 15) that is reminiscent of LDs that remain enveloped within the ER of *ScFIT* mutants [92].

The complexity of lipid metabolic pathways is underscored by the highly interconnected conversion of intermediates as well as the various organelles and proteins that mediate these

processes [1, 233]. In addition to this, perturbation of the lipid metabolic pathways results in the extensive reprogramming of the bioenergetic network [234, 235]. Similarly, cellular insults also alter the lipidomic landscape of cells, suggestive of the buffering capacity of lipid pathways against stress conditions. Proteotoxic or lipid bilayer stress both activate the UPR and similarly culminate in the formation of LDs. Interestingly, none of the hitherto major protein effectors of LD biogenesis were identified as UPR targets. Moreover, apart from the SE biosynthetic *ARE2*, no other NL synthesis players are upregulated under conditions of ER stress [113, 137]. Given the recent report of *Scs3* as a lipid phosphatase, its consequent upregulation under the UPR programme could be part of the effort to orchestrate membrane remodeling.

It was reported that LDs in *ScFITΔ* cells remain embedded in the ER due to the enrichment of DG, a lipid species with negative membrane curvature, as is the case with the accumulation of PE in a mutant of *CHO2*, a methyltransferase for PC synthesis [95]. Interestingly, the addition of either of the positive curvature phospholipids, lyso-PC or lyso-phosphatidic acid (PA), rescued the aberrant LD budding of *ScFITΔ* cells. The failure of the UPR to restore proper LD maturation in *cho2Δ* cells may result from the markedly reduced capacity to synthesise PC, which exhibits a neutral curvature and is an intermediate to lyso-PC [20]. While *cho2Δ* cells indeed accumulated high levels of PE, DG levels are dramatically reduced [113]. This taken together with the observation that the gain-of-function DG-binding FIT mutant failed to rescue the aberrant ER membrane whorling in *scs3Δ* cells [94] strongly suggest that defects in ER membrane properties that led to impaired LD maturation may be independent from DG. While the catalytic activity of mammalian FIT2 on PA and lyso-PA was not identified in ScFIT proteins [94, 97], this does not exclude the possibility that the latter may instead act on other membrane lipid species *in vivo*. It can then be hypothesised that *Scs3* regulates ER membrane lipid composition, presumably through the conversion of phospholipid species, to maintain proper ER function including LD maturation.

Insults to ER membrane homeostasis have been shown to not only activate the cognate UPR programme, but also increase the formation of LDs albeit with a poorly understood rationale

[113, 159]. The altered membrane morphology and consequent membrane tethering of LDs may be the synergistic effect of gross changes to membrane lipids and the absence of the robust buffering capacity of the UPR, as the result of the loss of *SCS3* and *IRE1*, respectively [94, 158]. It can be noted that two membrane-perturbed yeast mutants that are characterised by UPR activation have thus far been shown to prevent proper LD maturation [92, 95]. The *cho2Δ* mutant constitutively activates the UPR resulting from membrane lipid perturbation [113], but the membrane stress response alone was not sufficient to rescue the LD-ER tethering phenotype. This may be due to the stress condition arising from the absence of the same proteins that are required to restore membrane homeostasis (i.e. *CHO2* or *SCS3*). Whether or not LDs that arise from proteotoxic-UPR activation are similarly bound to the ER is a gap that needs to be addressed to completely ascertain the contribution of the UPR in membrane remodeling under such stress conditions.

The deletion mutant of *SCS3* was characterised early on as an inositol auxotroph [96], and this was later described to be the result of Opi1 mislocalization [97]. High levels of PA within the ER membrane drives the ER-localization of Opi1 in conjunction with Scs2 [236, 237]. Transcriptional regulation of phospholipid biosynthesis by Opi1 is highly dependent on Opi1 localization dynamics, and its release from the ER membrane and into the nucleus subsequently represses the expression of phospholipid biosynthesis by inhibiting the Ino2/4 transcriptional complex [238, 239]. The Pah1 phosphatidic acid phosphatase has been identified to regulate Opi1 binding to the ER by catalysing the conversion of PA to DAG [240, 241]. From this it can be hypothesised that the phosphatase activity of ScFIT might similarly influence PL metabolism. In support of this contention, high-throughput genetic interaction studies show that concurrent deletion of phospholipid biosynthetic repressors, including Opi1, improved the growth defect of *ScFITΔ* cells [89, 242]. However, no discernible PA-specific phosphatase activity has been detected in either of the ScFIT proteins [95], thereby suggesting that they may regulate Opi1 activity in a PA-independent manner. Although substrates for the putative function ScFIT have yet to be identified, its catalytic activity as a lipid phosphatase should nonetheless yield free phosphates [243]. Apart from the presence of PA, inositol, or choline, the repressive function of Opi1 has been shown to be reduced by

cellular phosphate levels in conjunction with the phosphate sensor Pho85 [244]. This may provide an alternative avenue for ScFIT-mediated regulation of phospholipid biosynthesis that warrants further investigation.

The UPR has long been identified to be intimately associated with inositol regulation, as both coordinately uphold ER functionality and structure through the synthesis of resident proteins and membrane lipids, respectively [245]. Since then, the adaptive membrane expansion mediated by the UPR has been described to be potentiated by *de novo* phospholipid synthesis through the Ino2/4 heterodimeric transcriptional complex [206]. However, stress-induced UPR and inositol response have mostly been studied as two discontinuous pathways [137, 246], and details on the cascade of protein effectors that culminate in the upregulation of phospholipid biosynthesis under UPR activation needs further elucidation. The *scs3Δ* mutant is one of the few phospholipid biosynthetic mutants that exhibit inositol auxotrophy, along with *ino2Δ* and *ino4Δ* [247]. The same auxotrophic phenotype was identified in the UPR-deficient mutants of *IRE1* and *HAC1*, strongly indicative of the UPR functioning in phospholipid biosynthesis [181, 248]. Although an overlap in target genes between the UPR and inositol response have been identified, distinct transcriptional regulatory motifs are present that differentiate the two, with the UPRE found in the promoter regions of UPR targets and the inositol-sensitive upstream activating sequence (UAS_{INO}) in those of phospholipid biosynthetic genes. How activation of the UPR is capable of concurrently upregulating UAS_{INO}-bearing gene targets, such as *OPI3* and *INO1*, in addition to its own warrants the investigation of protein factors that coordinate these two cellular pathways [137]. In support of this, the modularity in UPR-dependent gene regulation was demonstrated by the robust expression of a UPRE-driven reporter under secretory stress without a significant upregulation of a co-expressed *INO1* reporter [249]. Mutants of *SCS3* display the same sensitivity to secretory stress as that of *ire1Δ* and *hac1Δ*, and whether this is related to their functioning in inositol and phospholipid regulation has yet to be determined. However, it is conceivable that *Scs3*, as a UPR target gene, may function as the linchpin that bridges the UPR and the consequent phospholipid synthesis by modulation of Opi1 and the Ino2/4 transcriptional complex in a stress-dependent manner [89].

4.2 Membrane aberrations in *ScFIT* mutants negatively impacts protein quality control

Interestingly, our experimental data strongly suggest that defects associated with the absence of ScFIT proteins extend to processes outside of LD formation and phospholipid metabolism. The loss of both FIT homologs in *ScFITΔ* mutants led to the unexpected stabilisation of misfolded proteins both within the ER (Figure 21) and in the cytoplasm (Figure 23). Our results also suggest that this is not due to improper localization of client substrates, but rather to a global decrease in protein ubiquitination (Figure 22) and decrease in the extent of activation of adaptive proteostatic responses in *ScFITΔ* cells (Figure 11A, Figure 25).

The maintenance of ER membrane integrity and lipid homeostasis are critical in supporting organellar function. Loss of *ICE2* exhibits altered ER membrane dynamics including defects in mother-daughter cell ER membrane inheritance and ER-plasma membrane tethering [83, 84]. This ER membrane perturbation further impaired cellular functions such as phospholipid regulation and protein degradation [85, 207, 250, 251]. Similarly, mutants of the phospholipase Lpl1, which catalyses the turnover of phospholipids, also exhibited ERAD defects [70, 252]. The general disruption of lipid metabolism by attenuating FA synthesis in turn caused defects in processing of ERAD client proteins in mammalian systems [178], which may also be in part due to its indirect effects on membrane lipid composition. Conversely, defective protein turnover also exerted a direct effect on membrane composition. The deletion of the ERAD component *UBX2* led to severe changes in ER membrane morphology due to dysregulation of Mga2 processing and the subsequent expression of its transcriptional target *OLE1*, a key regulator of membrane lipid saturation [208]. Sterol content within membranes are also under tight control by protein quality control pathways, as the key enzymes Hmg2 and Erg1 are regulated in an ERAD-dependent mechanism [253, 254]. Intriguingly, these mutants with aberrant membranes exhibited impaired LD formation in addition to curtailed protein turnover [70, 85, 86].

Moreover, heat or proteotoxic and lipid bilayer stress activate the HSR and UPR, respectively, and similarly culminate in the formation of LDs [113, 159, 162]. This raises the question of whether or not LDs exert a relevant function in homeostatic pathways. While LDs were initially

thought to serve as temporary depots for client proteins marked for degradation [43, 255], cellular degradation pathways remained fully intact in mutants devoid of LDs [177, 178]. Furthermore, proteotoxic stress-induced LD formation persisted in the absence of *IRE1*, and LD-null cells remain viable under protein stress [159]. It is then possible their accumulation is a secondary effect of metabolic rewiring brought about by cellular insults [163].

In a previous study, we have shown that the singular UPR transducer Ire1 in yeast is strongly activated with genetic alterations of ER membrane composition [113]. An amphipathic helix within Ire1 is thought to drive its oligomerization and subsequent activation under membrane stress by forming energetically favorable localised membrane compression [132]. As ER membrane morphology is compromised in ScFIT mutants, it could be hypothesised that proper Ire1 oligomerization and UPR activation may not proceed as efficiently, which in turn affects ERAD function. In line with this, several studies lend support for the modulation of LDs by protein quality control pathways including ERAD. The *ubi4Δ* mutant was reported to exhibit less LD accumulation compared to that of WT cells under stress [162]. While the rationale for this increase in LD remains enigmatic, it suggests that LD formation may in part be regulated by ubiquitination processes. This agrees with previous studies that detailed on the dependence of the NL biosynthetic enzyme Dga1 and SE lipase Yeh1 on Doa10 for their endogenous turnover [47], and that recruitment of the mammalian ERAD factor UBXD8 onto the LD surface regulates LD growth by modulating lipolysis [256].

Apart from LDs, ERAD pathways also regulate the ER membrane composition and phospholipid turnover. The Cdc48 ATPase mediates the processing of the ER membrane sensors Mga2 and Spt24, to yield the cognate transcription factor for *OLE1* regulation [208, 257, 258], and the degradation of phosphorylation-inactive Pah1 is impaired in proteasome and ubiquitination mutants [73, 259]. Taken together, these greatly emphasises the interdependence of membrane homeostasis and protein quality control pathways.

While the decrease in the turnover of ERAD substrates can correspondingly extend to that of misfolded cytoplasmic proteins due to the similar dependence on the UPS system, the

substantial downregulation of the cytosolic HSR observed in *ScFITΔ* mutants is unexpected. Evidences of crosstalk between the UPR and HSR have been reported by several groups. HSR activation was shown to rescue protein-associated defects present in *ire1Δ* mutants [260], and facilitate clearance of a known ERAD substrate [261]. Conversely, the absence of either *IRE1* or *HAC1* strongly attenuated HSR activation [262]. Interestingly, the growth defect associated with inositol auxotrophy in *scs3Δ* mutants was found to be exacerbated at 37°C [247]. These then suggest that the loss of function of the ScFIT proteins in the adaptive UPR programme may similarly impair the HSR, thereby leading to the concurrent loss of two critical proteostatic response pathways.

Taken together, our study builds on the current hypothesis on the role of ScFIT proteins in LD formation and membrane homeostasis, and further provide support for its functioning in cell stress response pathways to exert effects on these two processes. (Figure 27).

Figure 27. Model for the putative role of ScFIT proteins in coordinating lipid and protein homeostasis

The ScFIT proteins functions in membrane homeostasis by preventing aberrant TG accumulation within the ER, thereby contributing to LD maturation. High levels of TG within the ER membrane may impair protein turnover. Alternatively, ScFIT proteins may directly modulate proteostatic pathways, such as the UPR and HSR, to aid in this process with an unknown mechanism. LD, lipid droplet; TG, triacylglycerol.

Chapter 5. Conclusion and Future Work

The FIT class of proteins has gained substantial scientific interest in recent years due to its association with various cellular defects and gross physiological dysfunctions [87, 263], and concerted efforts have since then begun to unravel their molecular function. Although the observed phenotypes upon the loss of FIT proteins and the severity of these defects vary between model systems, the evident evolutionary and functional conservation emphasises the their importance both in organismal development and in the maintenance of proper cellular fitness [100, 264]. However, the cascade of events by which the loss of FIT proteins give rise to such dysfunctional states remains elusive. Definitive information on the interactors of FIT proteins has been scarce if not inconclusive, and as a result has greatly limited the breadth of investigation on cellular pathways in which they may exert their functions. Moreover, while there are several existing hypotheses in the field describing its activity in lipid partitioning and phospholipid modification, how these putative functions could directly impact normative cellular processes has been a largely unaddressed scientific gap. In this study, we described the importance of *Scs3*, one of the *ScFIT* proteins, in the maintenance of ER membrane homeostasis in conjunction with the UPR, and how the concurrent loss of both factors led to an aberrant ER morphology that prevented the complete physical scission of LDs from the ER membrane. Furthermore, we demonstrated the unprecedented failure of *ScFITΔ* mutants to mount fully functional cell stress response pathways, namely the UPR and HSR, and its consequential disruption of protein turnover.

The UPR and HSR, apart from being stress adaptive cellular mechanisms, are intricately regulated to support optimal function and viability. It is also noteworthy that the phenotype associated with *FIT2* loss are, to a certain extent, regulated by these signaling pathways. The highly conserved IRE1 axis has been shown to coordinate the expression of adipocyte differentiation factors [265], as does the temperature-dependent induction of the HSR [266]. It is then highly possible that the impaired adipogenesis observed in *FIT2*-deficient cells is the result of disrupted stress response activation, rather than a direct function in lipogenic pathways [263]. The various physiological impairments in individuals with mutated in *FIT2* in the absence of lipodystrophic features also suggest of the importance of FIT proteins outside

the context of lipid metabolism [264]. The results of this study then warrant the investigation of FIT protein function in association with cell stress response regulation to gain a better understanding of its broad involvement in disease pathology.

5.1 Characterizing the functional relationship of ScFIT proteins in stress response activation and the coordination of phospholipid biosynthesis

We have reported on the significant downregulation of the HSR and UPR in the absence of the ScFIT proteins. However, the mechanism for this downregulation remains unknown. Hsf1 is the sole transcriptional activator of the HSR in yeast. *FIT2* deletion yielded no effect on the activation of the mammalian IRE1, while an increase in that of the alternative ATF4 axis of the UPR was reported [94]. This suggests that the loss of the FIT proteins could potentially prevent the proper activation of Ire1 in specific, potentially due to gross membrane alterations [132]. To further support this finding, oligomerization studies of Ire1 may be done [267] in addition to measuring the extent of splicing of the downstream *HAC1* transcription factor mRNA. HSR activation in yeast is dependent on the essential Hsf1 protein. As it is constitutively nuclear in localization as opposed to its dynamic mammalian counterpart, the extent of its phosphorylation may be used as an alternative to assess its activity [150]. In addition to these, transcriptomic analyses of UPR and HSR target genes in *ScFITΔ* mutants under basal conditions and/or in response to stress would provide invaluable insight into the importance of ScFIT protein function in supporting normal cell growth as well as in adaptive responses.

The ScFIT proteins have been extensively reported to be involved in phospholipid metabolism by several studies [89, 94, 97]. Moving forward, the identification of bona fide catalytic substrates of the ScFIT proteins would be critical in further understanding their relevance in ER membrane homeostasis. Furthermore, the various genetic interactions reported between *ScFIT* genes and those of phospholipid biosynthetic enzymes should be further characterised. This could be done through lipidomic and transcriptomic analyses of digenic or trigenic mutants. Lastly, the aberrant localization of Opi1 in the absence of ScFIT proteins suggests of its direct contribution to the transcriptional regulation of *de novo* phospholipid synthesis. As

there is currently no established link on how the UPR coordinates the expression of phospholipid genes, *Scs3* could possibly be the factor that bridges the two. In line with this, an *INO1* promoter-driven reporter expressed in *scs3Δ* or *ScFITΔ* mutants under ER stress conditions should be utilised to assess whether cells still exhibit the same UPR-induced expression of phospholipid biosynthetic genes in the absence of the ScFIT proteins [249].

5.2 Identifying key ERAD and UPS defects in *ScFIT* mutants

While common transcriptional targets are present between the two proteostatic cell response pathways, they primarily regulate distinct components of ERAD and the UPS. The UPR upregulates ERAD factors [137] while the HSR is responsible for regulating the abundance of free ubiquitin and proteasomal subunits [143, 144]. Given the inability of *ScFITΔ* mutants to effectively activate these cellular responses either basally or under stress conditions, the failure to degrade aberrantly folded proteins could be the result of the reduction in proteasomal degradation capacity. As ubiquitin-mediated degradation involves several interconnected components, the identification of factors that are directly affected by the loss of ScFIT proteins would be crucial in narrowing down which specific process is impaired in these mutants. The downregulation of the HSR under basal conditions in *ScFITΔ* mutants may lead to a decline in the protein levels of Ydj1 [149], a chaperone that has been reported to be critical in the turnover of misfolded substrates [175, 268]. Furthermore, the concurrent loss of Ubx2 aggravates the growth defect of *Scs3* mutants [89]. One possible hypothesis to be explored is that this exacerbates the inability of mutants to maintain proteostasis, and that this could be a feedforward loop that not only induces protein stress but also leads to membrane disruption [208, 257, 258]. To this end, large scale proteomic analyses (e.g. SILAC) may be employed to determine not only the identity of down/upregulated proteins, but also the extent of such a change in the absence of ScFIT proteins.

Several assays may additionally be performed to complement this investigation. We have shown a marked reduction in global protein ubiquitination in *ScFITΔ* mutants. However, ubiquitin chain linkages exist in several forms which can yield different effects, one of which is protein degradation signaling [269]. To ascertain that this ubiquitination defect is relevant in

the impairment of protein degradation, a substrate ubiquitination assay could be employed. This will not only inform on whether compromised protein ubiquitination is indeed the cause of its stabilization but could also suggest that ubiquitination factors may be affected in *ScFITΔ* mutants. We have also reported the physical interaction between Scs3 and the major E3 ligase Doa10, but the functional relevance of such an interaction has yet to be explored. At this point, the possibility that Scs3 may act in concert with Doa10 in protein ubiquitination could not be dismissed. However, we may not exclude the possibility that Scs3 itself is degraded in a Doa10-dependent manner. To address the latter, a comparative cycloheximide chase assay using epitope-tagged Scs3 should be done in WT and *doa10Δ* mutant yeast backgrounds. To further determine whether Scs3 directly functions in ERAD, the stability of short-lived misfolded Doa10 substrates could be monitored in *scs3Δ* or *ScFITΔ* yeast mutants. Although Scs3 contains no known ubiquitin binding domain, it may alternatively serve as an adaptor protein for ubiquitinated substrates *en route* to the proteasome, analogous to the reported function of Ubx2 [220]. In line with this, the capacity of Scs3 to bind ubiquitinated proteins may be investigated by co-immunoprecipitation followed by immunoblotting with ubiquitin-specific antibodies.

Appendix

Table S1. Frequency of ScFIT protein interactors identified through the membrane yeast two-hybrid screen. Functional descriptions are from the *Saccharomyces Genome Database* (SGD).

Protein	Frequency				Category	Description
	C _{ub} -Scs3	Scs3-C _{ub}	C _{ub} -Yft2	Yft2-C _{ub}		
Act1	1				Other Functions	Actin; structural protein involved in cell polarization, endocytosis, and other cytoskeletal functions
Adh1	1				Small Molecule Metabolism	Alcohol dehydrogenase; fermentative isozyme active as homo- or heterotetramers; required for the reduction of acetaldehyde to ethanol, the last step in the glycolytic pathway; ADH1 has a paralog, ADH5, that arose from the whole genome duplication
Alg2		1			Protein Processing	Mannosyltransferase in the N-linked glycosylation pathway; catalyzes two consecutive steps in the N-linked glycosylation pathway; alg2 mutants exhibit temperature-sensitive growth and abnormal accumulation of the lipid-linked oligosaccharide Man2GlcNAc2-PP-Dol; human ALG2 complements the temperature sensitivity and dolichol-linked oligosaccharide biosynthesis defect of the alg2-1 mutant, but mutant form from a patient with CDG-li fails to complement
Alg3		1			Protein Processing	Dolichol-P-Man dependent alpha(1-3) mannosyltransferase; involved in synthesis of dolichol-linked oligosaccharide donor for N-linked glycosylation of proteins; G353A missense mutation in human ortholog ALG3 implicated in carbohydrate deficient glycoprotein syndrome type IV, which is characterized by microcephaly, severe epilepsy, minimal psychomotor development,

						partial deficiency of sialic acids in serum glycoproteins; wild-type human ALG3 can complement yeast alg3 mutant
Atg22		2			Vacuolar Function	Vacuolar integral membrane protein required for efflux of amino acids; required for efflux of amino acids during autophagic body breakdown in the vacuole; null mutation causes a gradual loss of viability during starvation
Avt1	1			3	Vacuolar Function	Vacuolar transporter; imports large neutral amino acids into the vacuole; member of a family of seven <i>S. cerevisiae</i> genes (AVT1-7) related to vesicular GABA-glycine transporters
Bap2		1			Transmembrane Transport	High-affinity leucine permease; functions as a branched-chain amino acid permease involved in uptake of leucine, isoleucine and valine; contains 12 predicted transmembrane domains; BAP2 has a paralog, BAP3, that arose from the whole genome duplication
Cab3	1				Small Molecule Metabolism	Subunit of PPCDC and CoA-SPC complexes involved in CoA biosynthesis; subunits of the phosphopantothienoylcysteine decarboxylase (PPCDC) complex are: Cab3p, Sis2p, Vhs3p, while the subunits of the CoA-synthesizing protein complex (CoA-SPC) are: Cab2p, Cab3p, Cab4p, and Cab5p as well as Sis2p and Vhs3p; null mutant lethality is complemented by <i>E. coli</i> coaBC
Chs1		1			Cell Wall	Chitin synthase I; requires activation from zymogenic form in order to catalyze the transfer of N-acetylglucosamine (GlcNAc) to chitin; required for repairing the chitin septum during cytokinesis; transcription activated by mating factor
Cpr6	1	1			Other Functions	Peptidyl-prolyl cis-trans isomerase (cyclophilin); catalyzes the cis-trans isomerization of peptide bonds N-terminal to proline

						residues; plays a role in determining prion variants; binds to Hsp82p and contributes to chaperone activity; protein abundance increases in response to DNA replication stress
Cyb5		2			Lipid Metabolism	Cytochrome b5; involved in the sterol and lipid biosynthesis pathways; acts as an electron donor to support sterol C5-6 desaturation
Doa10		3			Protein Processing	Membrane-embedded ubiquitin-protein ligase; ER and inner nuclear membrane localized RING-CH domain E3 ligase involved in ER-associated protein degradation (ERAD); targets misfolded cytosolic/nucleoplasmic domains of soluble and membrane embedded proteins (ERAD-C) and a transmembrane domain containing substrate (ERAD-M), Sbh2p; C-terminal element (CTE), conserved in human ortholog MARCH10/TEB4, determines substrate selectivity
Dps1	1				Cytoplasmic Translation	Aspartyl-tRNA synthetase, primarily cytoplasmic; homodimeric enzyme that catalyzes the specific aspartylation of tRNA(Asp); class II aminoacyl tRNA synthetase; binding to its own mRNA may confer autoregulation; shares five highly conserved amino acids with human that when mutated cause leukoencephalopathy characterized by hypomyelination with brain stem and spinal cord involvement and leg spasticity (HBSL)
Ecm4	1				Other Functions	S-glutathionyl-(chloro)hydroquinone reductase (GS-HQR); glutathione transferase involved in cell-surface biosynthesis and architecture; catalyzes glutathione (GSH)-dependent reduction of GS-trichloro-p-hydroquinone to trichloro-p-hydroquinone;

						expression upregulated upon exposure to genotoxic agents, such as methyl methanesulfonate, cisplatin and bleomycin; not an essential gene; similar to YGR154C; green fluorescent protein (GFP)-fusion protein localizes to cytoplasm
Emp24		1			Endomembrane Transport	Component of the p24 complex; role in misfolded protein quality control; binds to GPI anchor proteins and mediates their efficient transport from the ER to the Golgi; integral membrane protein that associates with endoplasmic reticulum-derived COPII-coated vesicles
Ena1		2			Transmembrane Transport	P-type ATPase sodium pump; involved in Na ⁺ and Li ⁺ efflux to allow salt tolerance
Ept1	1				Lipid Metabolism	sn-1,2-diacylglycerol ethanolamine- and cholinephosphotransferase; not essential for viability; EPT1 has a paralog, CPT1, that arose from the whole genome duplication
Erg1		1			Lipid Metabolism	Squalene epoxidase; catalyzes the epoxidation of squalene to 2,3-oxidosqualene; plays an essential role in the ergosterol-biosynthesis pathway and is the specific target of the antifungal drug terbinafine; human SQLE functionally complements the lethality of the erg1 null mutation
Fks1		5			Cell Wall	Catalytic subunit of 1,3-beta-D-glucan synthase; functionally redundant with alternate catalytic subunit Gsc2p; binds to regulatory subunit Rho1p; involved in cell wall synthesis and maintenance; localizes to sites of cell wall remodeling; FKS1 has a paralog, GSC2, that arose from the whole genome duplication
Flr1		1			Transmembrane Transport	Plasma membrane transporter of the major facilitator superfamily; member of the 12-spanner drug:H(+) antiporter DHA1

						family; involved in efflux of fluconazole, diazaborine, benomyl, methotrexate, and other drugs; expression induced in cells treated with the mycotoxin patulin; relocates from nucleus to plasma membrane upon DNA replication stress
Gnp1		1		1	Transmembrane Transport	High-affinity glutamine permease; also transports Leu, Ser, Thr, Cys, Met and Asn; expression is fully dependent on Grr1p and modulated by the Ssy1p-Ptr3p-Ssy5p (SPS) sensor of extracellular amino acids; GNP1 has a paralog, AGP1, that arose from the whole genome duplication
Gpb2	1				Cytoplasmic Translation	Poly(A+) RNA-binding protein; key surveillance factor for the selective export of spliced mRNAs from the nucleus to the cytoplasm; preference for intron-containing genes; similar to Npl3p; also binds single-stranded telomeric repeat sequence in vitro; relocates to the cytosol in response to hypoxia; GBP2 has a paralog, HRB1, that arose from the whole genome duplication
Gsc2		3			Cell Wall	Catalytic subunit of 1,3-beta-glucan synthase; involved in formation of the inner layer of the spore wall; activity positively regulated by Rho1p and negatively by Smk1p; GSC2 has a paralog, FKS1, that arose from the whole genome duplication
Hht2	1				Other Functions	Histone H3; core histone protein required for chromatin assembly, part of heterochromatin-mediated telomeric and HM silencing; one of two identical histone H3 proteins (see HHT1); regulated by acetylation, methylation, and phosphorylation; H3K14 acetylation plays an important role in the unfolding of strongly positioned nucleosomes during repair of UV damage
Hom2	1				Small Molecule	Aspartic beta semi-aldehyde

					Metabolism	dehydrogenase; catalyzes the second step in the common pathway for methionine and threonine biosynthesis; expression regulated by Gcn4p and the general control of amino acid synthesis
Hom6	1				Small Molecule Metabolism	Homoserine dehydrogenase (L-homoserine:NADP oxidoreductase); dimeric enzyme that catalyzes the third step in the common pathway for methionine and threonine biosynthesis; enzyme has nucleotide-binding, dimerization and catalytic regions
Hta2	1				Other Functions	Histone H2A; core histone protein required for chromatin assembly and chromosome function; one of two nearly identical (see also HTA1) subtypes; DNA damage-dependent phosphorylation by Mec1p facilitates DNA repair; acetylated by Nat4p
Hxk1	1				Small Molecule Metabolism	Hexokinase isoenzyme 1; a cytosolic protein that catalyzes phosphorylation of glucose during glucose metabolism; expression is highest during growth on non-glucose carbon sources; glucose-induced repression involves hexokinase Hxk2p; HXK1 has a paralog, HXK2, that arose from the whole genome duplication
Hxt1		1			Transmembrane Transport	Low-affinity glucose transporter of the major facilitator superfamily; expression is induced by Hxk2p in the presence of glucose and repressed by Rgt1p when glucose is limiting; HXT1 has a paralog, HXT6, what arose from the whole genome duplication
Hxt3		1	21	3	Transmembrane Transport	Low affinity glucose transporter of the major facilitator superfamily; expression is induced in low or high glucose conditions; HXT3 has a paralog, HXT5, that arose from the whole genome duplication
Hxt4		1			Transmembrane Transport	High-affinity glucose transporter; member of the major facilitator superfamily,

						expression is induced by low levels of glucose and repressed by high levels of glucose; HXT4 has a paralog, HXT7, that arose from the whole genome duplication
Izh3		1			Other Functions	Membrane protein involved in zinc ion homeostasis; member of the four-protein IZH family, expression induced by zinc deficiency; deletion reduces sensitivity to elevated zinc and shortens lag phase, overexpression reduces Zap1p activity
Kes1	1				Endomembrane Transport	One of seven members of the yeast oxysterol binding protein family; involved in negative regulation of Sec14p-dependent Golgi complex secretory functions, peripheral membrane protein that localizes to the Golgi complex; KES1 has a paralog, HES1, that arose from the whole genome duplication
Lyp1		2			Transmembrane Transport	Lysine permease; one of three amino acid permeases (Alp1p, Can1p, Lyp1p) responsible for uptake of cationic amino acids
Mrh1				3	Other Functions	Protein that localizes primarily to the plasma membrane; also found at the nuclear envelope; long-lived protein that is asymmetrically retained in the plasma membrane of mother cells; the authentic, non-tagged protein is detected in mitochondria in a phosphorylated state; null mutation confers sensitivity to acetic acid
Nop56	1				Other Functions	Essential evolutionarily-conserved nucleolar protein; component of the box C/D snoRNP complexes that direct 2'-O-methylation of pre-rRNA during its maturation; overexpression causes spindle orientation defects
Ost4				2	Protein Processing	Subunit of the oligosaccharyltransferase complex of the ER lumen; complex catalyzes protein asparagine-linked glycosylation; type I membrane protein required

						for incorporation of Ost3p or Ost6p into the OST complex
Pcd1	1				Small Molecule Metabolism	8-oxo-dGTP diphosphatase; prevents spontaneous mutagenesis via sanitization of oxidized purine nucleoside triphosphates; can also act as peroxisomal pyrophosphatase with specificity for coenzyme A and CoA derivatives, may function to remove potentially toxic oxidized CoA disulfide from peroxisomes to maintain the capacity for beta-oxidation of fatty acids; nudix hydrolase family member; similar E. coli MutT and human, rat and mouse MTH1
Pdr5		3			Transmembrane Transport	Plasma membrane ATP-binding cassette (ABC) transporter; multidrug transporter actively regulated by Pdr1p; also involved in steroid transport, cation resistance, and cellular detoxification during exponential growth; PDR5 has a paralog, PDR15, that arose from the whole genome duplication
Pet9		2			Transmembrane Transport	Major ADP/ATP carrier of the mitochondrial inner membrane; exchanges cytosolic ADP for mitochondrially synthesized ATP; also imports heme and ATP; required for viability in many lab strains that carry a sal1 mutation; PET9 has a paralog, AAC3, that arose from the whole genome duplication; human homolog SLC25A4 implicated in progressive external ophthalmoplegia can complement yeast null mutant
Pgk1	4				Small Molecule Metabolism	3-phosphoglycerate kinase; catalyzes transfer of high-energy phosphoryl groups from the acyl phosphate of 1,3-bisphosphoglycerate to ADP to produce ATP; key enzyme in glycolysis and gluconeogenesis
Pho3	1				Other Functions	Constitutively expressed acid phosphatase similar to Pho5p; brought to the cell surface by transport vesicles;

						hydrolyzes thiamin phosphates in the periplasmic space, increasing cellular thiamin uptake; expression is repressed by thiamin
Pho88		4		18	Transmembrane Transport	Protein involved in SRP-independent targeting of substrates to the ER; component of an alternative ER targeting pathway that has partial functional redundancy with the GET pathway; preference for substrates with downstream transmembrane domains; interacts with Snd1p, Env10p/Snd2p, and Sec61p-translocon subunits; can compensate for loss of SRP; role in phosphate transport, interacting with pho88, and in the maturation of secretory proteins
Pma1		3			Transmembrane Transport	Plasma membrane P2-type H ⁺ -ATPase; pumps protons out of cell; major regulator of cytoplasmic pH and plasma membrane potential; long-lived protein asymmetrically distributed at plasma membrane between mother cells and buds; accumulates at high levels in mother cells during aging, buds emerge with very low levels of Pma1p, newborn cells have low levels of Pma1p; Hsp30p plays a role in Pma1p regulation; interactions with Std1p appear to propagate [GAR+]
Pmr1		3			Transmembrane Transport	High affinity Ca ²⁺ /Mn ²⁺ P-type ATPase; required for Ca ²⁺ and Mn ²⁺ transport into Golgi; involved in Ca ²⁺ dependent protein sorting, processing; D53A mutant (Mn ²⁺ transporting) is rapamycin sensitive, Q783A mutant (Ca ²⁺ transporting) is rapamycin resistant; Mn ²⁺ transport into Golgi lumen required for rapamycin sensitivity; mutations in human homolog ATP2C1 cause acantholytic skin condition Hailey-Hailey disease; human ATP2C1 can complement yeast null mutant

Psa1	1				Cell Wall	GDP-mannose pyrophosphorylase (mannose-1-phosphate guanylyltransferase); synthesizes GDP-mannose from GTP and mannose-1-phosphate in cell wall biosynthesis; required for normal cell wall structure
Ptm1		2	3		Unknown Function	Protein of unknown function; copurifies with late Golgi vesicles containing the v-SNARE Tlg2p; PTM1 has a paralog, YHL017W, that arose from the whole genome duplication
Ptr2		1		2	Transmembrane Transport	Integral membrane peptide transporter; mediates transport of di- and tri-peptides; conserved protein that contains 12 transmembrane domains; PTR2 expression is regulated by the N-end rule pathway via repression by Cup9p
Pup1	1				Protein Processing	Beta 2 subunit of the 20S proteasome; endopeptidase with trypsin-like activity that cleaves after basic residues; synthesized as a proprotein before being proteolytically processed for assembly into 20S particle; human homolog is subunit Z
Rme1	1				Other Functions	Zinc finger protein involved in control of meiosis; prevents meiosis by repressing IME1 expression and promotes mitosis by activating CLN2 expression; directly repressed by a1-alpha2 regulator; mediates cell type control of sporulation; relocates from nucleus to cytoplasm upon DNA replication stress
Rpl8b	1				Cytoplasmic Translation	Ribosomal 60S subunit protein L8B; required for processing of 27SA3 pre-rRNA to 27SB pre-rRNA during assembly of large ribosomal subunit; depletion leads to a turnover of pre-rRNA; L8 binds to Domain I of 25S and 5.8 S rRNAs; mutation results in decreased amounts of free 60S subunits; homologous to mammalian ribosomal protein L7A, no bacterial homolog;

						RPL8B has a paralog, RPL8A, that arose from the whole genome duplication
Rps17b				2	Cytoplasmic Translation	Ribosomal protein 51 (rp51) of the small (40s) subunit; homologous to mammalian ribosomal protein S17, no bacterial homolog; RPS17B has a paralog, RPS17A, that arose from the whole genome duplication; protein abundance increases in response to DNA replication stress
Rrp1		1			Other Functions	Essential evolutionarily conserved nucleolar protein; necessary for biogenesis of 60S ribosomal subunits and for processing of pre-rRNAs to mature rRNA; associated with several distinct 66S pre-ribosomal particles
Scs7		1			Lipid Metabolism	Sphingolipid alpha-hydroxylase; functions in the alpha-hydroxylation of sphingolipid-associated very long chain fatty acids, has both cytochrome b5-like and hydroxylase/desaturase domains, not essential for growth
Shr3			9	8	Endomembrane Transport	Endoplasmic reticulum packaging chaperone; required for incorporation of amino acid permeases into COPII coated vesicles for transport to the cell surface
Sit1		1			Transmembrane Transport	Ferrioxamine B transporter; member of the ARN family of transporters that specifically recognize siderophore-iron chelates; transcription is induced during iron deprivation and diauxic shift; potentially phosphorylated by Cdc28p
Slg1		1		1	Cell Wall	Sensor-transducer of the stress-activated PKC1-MPK1 kinase pathway; involved in maintenance of cell wall integrity; required for mitophagy; involved in organization of the actin cytoskeleton; secretory pathway Wsc1p is required for the arrest of secretion response
Ssb2	1				Cytoplasmic Translation	Cytoplasmic ATPase that is a ribosome-associated molecular chaperone;

						functions with J-protein partner Zuo1p; may be involved in the folding of newly-synthesized polypeptide chains; member of the HSP70 family; SSB2 has a paralog, SSB1, that arose from the whole genome duplication
Sti1	1				Other Functions	Hsp90 cochaperone; regulates spatial organization of amyloid-like proteins in the cytosol, thereby buffering the proteotoxicity caused by amyloid-like proteins; interacts with the Ssa group of the cytosolic Hsp70 chaperones and activates Ssa1p ATPase activity; interacts with Hsp90 chaperones and inhibits their ATPase activity; homolog of mammalian Hop
Stt3	1	4			Protein Processing	Subunit of the oligosaccharyltransferase complex of the ER lumen; complex catalyzes asparagine-linked glycosylation of newly synthesized proteins; forms a subcomplex with Ost3p and Ost4p and is directly involved in catalysis
Tat1		1			Transmembrane Transport	Amino acid transporter for valine, leucine, isoleucine, and tyrosine; low-affinity tryptophan and histidine transporter; overexpression confers FK506 and FTY720 resistance; protein abundance increases in response to DNA replication stress
Tdh1	1				Small Molecule Metabolism	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), isozyme 1; involved in glycolysis and gluconeogenesis; tetramer that catalyzes the reaction of glyceraldehyde-3-phosphate to 1,3 bis-phosphoglycerate; detected in the cytoplasm and cell wall; protein abundance increases in response to DNA replication stress; GAPDH-derived antimicrobial peptides secreted by <i>S. cerevisiae</i> are active against a wide variety of wine-related yeasts and

						bacteria
Tef2	2	1			Cytoplasmic Translation	Translational elongation factor EF-1 alpha; in the GTP-bound active form, binds to and delivers aminoacylated tRNA to the A-site of ribosomes for elongation of nascent polypeptides; associates with vacuolar Rho1p GTPase; TEF2-RFP levels increase during replicative aging; may also have a role in tRNA re-export from the nucleus; TEF2 has a paralog, TEF1, that arose from the whole genome duplication
Trr1	1				Other Functions	Cytoplasmic thioredoxin reductase; key regulatory enzyme that determines the redox state of the thioredoxin system, which acts as a disulfide reductase system and protects cells against both oxidative and reductive stress; protein abundance increases in response to DNA replication stress; TRR1 has a paralog, TRR2, that arose from the whole genome duplication
Uga4				2	Transmembrane Transport	GABA (gamma-aminobutyrate) permease; serves as a GABA transport protein involved in the utilization of GABA as a nitrogen source; catalyzes the transport of putrescine and delta-aminolevulinic acid (ALA); localized to the vacuolar membrane
Vma16		1		2	Vacuolar Function	Subunit c" of the vacuolar ATPase; v-ATPase functions in acidification of the vacuole; one of three proteolipid subunits of the V0 domain
Ydr524c-b				1	Unknown Function	Putative protein of unknown function; SWAT-GFP and mCherry fusion proteins localize to the endoplasmic reticulum; YDR524C-B has a paralog, YCL048W-A, that arose from the whole genome duplication
Ygr127w	1				Unknown Function	Putative protein of unknown function; expression is regulated by Msn2p/Msn4p, indicating a possible role in stress response

Yir014w		1			Unknown Function	Putative protein of unknown function; green fluorescent protein (GFP)-fusion protein localizes to the vacuole; expression directly regulated by the metabolic and meiotic transcriptional regulator Ume6p; YIR014W is a non-essential gene
Ynr061c		1		6	Unknown Function	Protein of unknown function; relocates from vacuole to cytoplasm upon DNA replication stress
Ypq2		1			Vacuolar Function	Putative vacuolar membrane transporter for cationic amino acids; likely contributes to amino acid homeostasis by exporting cationic amino acids from the vacuole; member of the PQ-loop family, with seven transmembrane domains; mutant phenotype is functionally complemented by rat PQLC2 vacuolar transporter
Zrc1		2		1	Vacuolar Function	Vacuolar membrane zinc transporter; transports zinc from cytosol to vacuole for storage; also has role in resistance to zinc shock resulting from sudden influx of zinc into cytoplasm; human ortholog SLC30A10 functions as a Mn transporter and mutations in SLC30A10 cause neurotoxic accumulation of Mn in liver and brain; ZRC1 has a paralog, COT1, that arose from the whole genome duplication
Zuo1	1				Cytoplasmic Translation	Ribosome-associated chaperone; zuotin functions in ribosome biogenesis and as a chaperone for nascent polypeptide chains in partnership with Ssz1p and SSb1/2; contains a DnaJ domain and functions as a J-protein partner for Ssb1p and Ssb2p; human gene DNAJC2 can partially complement yeast zuo1 null mutant

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