An intact IRE1 transmembrane domain is necessary for UPR activation upon lipid bilayer stress in *S. cerevisiae*

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**AN INTACT IRE1 TRANSMEMBRANE DOMAIN IS
NECESSARY FOR UPR ACTIVATION UPON LIPID BILAYER
STRESS IN *S. CEREVISIAE***

NURULAIN HO

SCHOOL OF BIOLOGICAL SCIENCES

2019

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STRESS IN *S. CEREVISIAE***

NURULAIN HO

SCHOOL OF BIOLOGICAL SCIENCES

Supervisor: Asst. Prof. Guillaume Thibault

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

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

25th August 2019

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The contributions of the co-authors are as follows:

- I prepared the manuscript drafts. The manuscript was revised by Dr Chengchao Xu.

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Summary

The unfolded protein response (UPR), a complex adaptive stress response of the endoplasmic reticulum (ER), is compromised in disease and aging. Typically activated by the accumulation of misfolded proteins in the ER, the UPR is similarly activated by alterations to ER fatty acids and lipid composition. Chronic UPR activation by ER lipid aberrations, which we term lipid-bilayer stress unfolded protein response (UPR^{LBS}) leads to the development of the metabolic syndrome non-alcoholic fatty liver disease (NAFLD) in obesity. Since critical insights into the pathophysiological mechanisms of the UPR in liver disease is unclear, we initiated a genome wide screen to determine biological processes involved in the buffering of this homeostatic response during UPR^{LBS}. An interesting gene that emerged from the screen as a key activator of the UPR upon its deletion is the methyltransferase Opi3, a phosphatidylcholine (PC) synthetic enzyme. It has been reported that an increase in endoplasmic reticulum PC ratio is observed in metabolic syndromes. Here, we show that the deletion of *OPI3* led to UPR^{LBS} activation independently of the luminal domain of Ire1 (Ire1 Δ LD). Our current research distinctly separates proteotoxic stress induced UPR (UPR^{PT}) and UPR^{LBS} suggesting that Ire1 offers novel and diverse mechanisms for the regulation of lipid homeostasis of the ER. The emerging potential of transmembrane domains in lipid sensing was confirmed when the mutation of the Ire1 residue R537Q ablated Ire1 downstream activity during LBS. An exciting outcome of our transcriptomic and ChIP-qPCR data reveal 3 novel genes *PBI2*, *PIR3* and *PUT1* upregulated solely by UPR^{LBS}. New insights into our data suggest that UPR^{LBS} is sensed and governed differently by Ire1 and an alternative transcriptional programme is activated for the adaptation of cells subject to lipid bilayer stress.

Abbreviations

Abbreviation	Description
4-PBA	4-phenylbutyrate
A	Ampere
ATF6	Activating transcription factor 6
BiFC	Bimolecular fluorescence complementation
BiP	Immunoglobulin heavy chain binding protein
BSA	Bovine serum albumin
bZIP	Basic leucine zipper domain
Ca ²⁺	Calcium
CDP DAG	Cytidine diphosphate diacylglycerol
ChIP	Chromatin immunoprecipitation
Cho	Choline
CHOP	CCAAT-enhancer-binding protein homologous protein
COPII	Coat protein complex II
CORVET	Class C core vacuole/endosome tethering
CSSR	Core stress sensing region
CYC1	Cytochrome c, isoform 1
DHC	Dihydroceramide
DHS	Dihydrosphingosine
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
eIF2 α	Eukaryotic translation initiation factor 2 α
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ERDjs	ER-localised DnaJ-like proteins
FA	Free fatty acid
FoxO1	Forkhead box O1
G	Relative centrifugal force
GADD34	Growth arrest and DNA damage-inducible protein
GFP	Green fluorescent protein
Hac1	Histone acetyltransferase 1

HEM1	5-aminolevulinate synthase
HgX	Hygromycin
His	Histidine
HSE	Heat shock element
Hsp	Heat shock protein
HS	Heat shock
HSR	Heat shock response
IF	Immunofluorescence
Ire1	Inositol requiring enzyme 1
JDP	J-domain containing co-chaperones
Kan	Genetic sulphate
L	Litre
LBS	Lipid bilayer stress
LD	Luminal domain
Leu	Leucine
LiCl	Lithium chloride
LiOAc	Lithium acetate
Met	Methionine
MHC	Major histocompatibility complex
NaCl	Sodium chloride
NAFLD	Non-alcoholic fatty liver disease
NBD	Nucleotide binding domain
NASH	Non-alcoholic steatohepatitis
NEFs	Nucleotide exchange factors
OD	Optical density
OE	Overexpression
ONPG	O-nitrophenyl- β -D-galactosidase
OS9	Osteosarcoma amplified 9
PA	Phosphatidic acid
PC	Phosphatidylcholine
PDI	Protein disulphide isomerase
PDME	Phosphodimethylethanolamine
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol

PEMT	Phosphatidylethanolamine methyltransferase
PERK	Protein Kinase RNA-like ER kinase
PGK1	3-phosphoglycerate kinase
PIC	Protease inhibitor cocktail
PLB	Protein loading buffer
PMME	Phosphomonomethylethanolamine
PMSF	Phenylmethylsulfonyl fluoride
PRC1	Vacuolar carboxypeptidase Y
PT	Proteotoxic stress
qPCR	Quantitative polymerase chain reaction
RIDD	Ire1-dependent decay of mRNA
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S. Pombe</i>	<i>Schizosaccharomyces pombe</i>
SBD	Substrate binding domain
SDS	Sodium dodecyl sulfate
SEM	Standard error mean
SERCA	Sarco/endoplasmic reticulum calcium ATPase
SFA	Saturated free fatty acid
SOC	Super optimal broth with catabolite repression
ssDNA	Salmon sperm DNA
T2D	Type 2 diabetes
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TD	Transmembrane domain
TDH3	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
TE	Tris-EDTA
TEF2	Translation elongation factor 2
Tm	Tunicamycin
TM	Transmembrane domain
tRNA	Transfer ribonucleic acid
Trp	Tryptophan

UPR	Unfolded protein response
UPRE	Unfolded protein response element
Ura	Uracil
UTR	Untranslated region
V	Voltage
WT	Wild-type
XBP1	Transcription factor X-box binding protein 1
YPD	Yeast extract-peptone-dextrose

Chapter 1. Introduction

The key factors in the unfolded protein response (UPR) were identified during the search for factors required for endoplasmic reticulum (ER)-to-nucleus communication [1-5]. The establishment of the UPR field was rather serendipitous, nevertheless it surprised the scientific community by setting up a paradigm of unconventional signal transduction. Since then, the field has been ever expanding, just like any other fundamental discovery. As a means of coping with misfolded proteins and restoring ER homeostasis, it is unsurprising that the UPR is directly linked to protein-misfolding diseases, such as Alzheimer's and Parkinson's [6]. Alternatively, cancer cells exploit the UPR to sustain their high demand for protein production, a prerequisite for unconditional proliferation [7]. Current evidence also shows a clear association between the UPR and the hallmarks of metabolic syndromes, dyslipidemia and obesity [8, 9]. Dyslipidemia, characterised as an increase in fasting and postprandial serum triglyceride (TAG) and cholesterol levels, is a common feature observed in obese patients [10, 11]. Obesity is now a global health pandemic afflicting individuals of the developing and developed countries [12]. Both obesity and dyslipidemia are contributing risk factors for metabolic diseases such as non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes (T2D). NAFLD is the most prevalent liver disease worldwide that is marked by the abnormal accumulation of fat in the liver [13]. The progression of NAFLD to severe non-alcoholic steatohepatitis (NASH) results in chronic inflammation of hepatocytes, cirrhosis of hepatic tissue and hepatocellular carcinoma, culminating in terminal liver damage [14]. Recent studies suggest that elevated levels of serum free saturated fatty acids, a consequence of dyslipidemia, play a role in the damage caused to hepatocytes during NAFLD [15]. Similarly, T2D has been associated with increased levels of triglycerides and free fatty acids eventually leading to β -cell dysfunction and insulin resistance [16-18]. Contrary to the well-established roles of the UPR in resolving protein misfolding, the UPR as a contributor of lipotoxic cell death in the liver and pancreatic β - cells are poorly characterised [19, 20]. This introduction will describe the classical functions of the UPR in maintaining ER protein homeostasis, before discussing the importance of lipid species in ER membrane homeostasis. The different types of membrane aberrancies affecting ER homeostasis mentioned in the following sections will be referred as lipid bilayer stress (LBS). We will discuss insights derived from the lower eukaryote *Saccharomyces cerevisiae* substantiated with evidence from mammalian *in vitro* and *in vivo* models. We will also examine the intricacy between lipid bilayer stress and the UPR, how the mechanism of activation

remains poorly understood and emphasise the implications that are relevant for disease progression for the importance of this study.

1.1 The unfolded protein response sensors

As an ancient counter-stress programme to ease the damaging effects in the ER, extensive efforts have been made to better understand the UPR. Typically, the accumulation of unfolded proteins in the ER activates the three ER-stress transducers, inositol-requiring enzyme 1 α (Ire1 α), PRKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6), resulting in translational control and transcriptional reprogramming of UPR target genes to elicit global cellular changes [21] (Figure 1).

Figure 1. Activation of the unfolded protein response (UPR). (A) The accumulation of unfolded proteins or lipid bilayer stress (LBS) in the ER activates three distinct UPR sensors. Upon ER stress monomeric Ire1 α and PERK1 form dimers or higher oligomers upon sensing stress followed by trans-autophosphorylation. Ire1 α ribonuclease domain cleaves the intron of *XBP1* mRNAs, and newly synthesized XBP1 activates the transcription of UPR target genes. Ire1 α may also initiate regulated Ire1-dependent decay (RIDD) of a specific subset of mRNA to decrease mRNA load. JNK and NF- κ B are activated by Ire1 and initiate the transcription of proinflammatory genes. Activated PERK phosphorylates eIF2 α , resulting in the temporary attenuation of general translation and the activation of the transcription factor ATF4, which subsequently activates the transcription of specific UPR target genes such as CHOP and GADD34 (shown in dark blue). CHOP is a pro-apoptotic factor that initiates cell death. CHOP is also linked to NF- κ B activation. Subsequently, GADD34 promotes the dephosphorylation of eIF2 α , resuming translation. When sensing unfolded proteins, the ATF6 luminal

domain stimulates its relocation to the Golgi apparatus where the transcription factor is released by intramembrane proteolysis. Soluble ATF6 then translocates to the nucleus where it upregulates the expression of a subset of UPR target genes. (B) *S. cerevisiae* Ire1 (Sclre1) undergoes dimerization or higher oligomerization and trans-autophosphorylation resulting in *HAC1* mRNA splicing through its RNase domain. Transcription factor Hac1 upregulates UPR target genes to restore homeostasis. (C) Activated *S. pombe* Ire1 (Splre1) triggers downstream RIDD activity similar to Ire1 α . LBS refers to lipid bilayer stress of the ER membrane; it triggers an activation mechanism indistinct from the conventional UPR activated by unfolded proteins in the ER. Figure reproduced with permission from [22].

Ire1 α drives the most evolutionary conserved branch of the UPR, also solely present in lower eukaryotes, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (referred in the introduction to as Sclre1 and Splre1 respectively for clarity) (Figure 1B, C). As a first step towards misfolded protein stress sensing, the N-terminal luminal domain (LD) of Ire1 α /Sclre1 binds to misfolded proteins through a dimeric interface peptide binding groove [23-25] (Figure 1A, B). There are two opposing, established models of Ire1 α /Sclre1 sensing by its luminal domain: the first model describes an indirect method of Ire1 α /Sclre1 luminal domain activation via the dissociation of the ER chaperone BiP, also known as Kar2 in yeast. This model is defined by the competitive titration of BiP/Kar2 from Ire1 α /Sclre1 in the presence of misfolded proteins. The second model suggests that Ire1 luminal domain directly recognises misfolded proteins in the endoplasmic reticulum, independently of BiP/Kar2. Most recently, a third BiP/Kar2 “allosteric” model of Ire1 activation was proposed.

BiP/Kar2, belonging to the Hsp70 family of chaperones, provides a major driving force for protein folding within the ER lumen. BiP/Kar2 plays important roles in ER quality control by participating in protein aggregation prevention, protein disaggregation, protein refolding and degradation [26, 27]. Characteristic of Hsp70 chaperones, BiP/Kar2 contains a N-terminal nucleotide binding domain (NBD) followed by a substrate binding domain (SBD) connected by a flexible linker [26] (Figure 2). The response of BiP/Kar2 to misfolded proteins is tightly regulated by an allosteric cycle of ATP binding, hydrolysis and nucleotide exchange. As the sole Hsp70 chaperone expressed abundantly within the ER lumen, BiP/Kar2 cycles through a typical Hsp70 chaperone-substrate mechanism that involves the recruitment of misfolded proteins to the SBD by J-domain containing co-chaperones (JDP) termed as ER-localised DnaJ-like proteins (ERdjs) [28] (Figure 2). This association by ERdjs greatly enhances the ATPase activity within the NBD, resulting in a closed conformation, high affinity state of BiP/Kar2 bound to adenosine diphosphate (ADP), trapping misfolded proteins within the SBD (Figure 2). Nucleotide

exchange factors (NEFs), catalysing the exchange of ADP to adenosine triphosphate (ATP), reverses BiP/Kar2 into a low affinity state resulting in the release of the bound protein substrate [26-28]. NEFs exchange ADP for ATP for substrate release and ensures the availability of BiP for another round of substrate binding [27, 28].

Figure 2: The ATPase cycle of BiP in the endoplasmic reticulum. BiP bound to ATP results in the nucleotide binding domain (NBD, grey) and substrate binding domain (SBD, blue) coming into close proximity resulting in a low affinity (ATP) binding state. The association of J-domain co-chaperones enhances the ATPase activity in the NBD resulting in a closed conformation high affinity (ADP) state, trapping misfolded proteins in the SBD. Figure reproduced from [29]

Besides its well-known role as a chaperone, BiP/Kar2 has been proposed to play a role in the negative regulation of Ire1 α /Scire1 [30]. The indirect method of activation is described as the competitive titration of BiP/Kar2 from Ire1 α /Scire1 when misfolded proteins accumulate in the ER lumen. BiP overexpression attenuated the UPR suggesting its negative regulation of Ire1 α activity [31]. This attenuation of the UPR was explained by BiP/Kar2 binding repressively to Ire1 LD via its SBD, keeping Ire1 in its monomeric form [30-34]. A J-domain containing co-chaperone ERdj4 associates to Ire1 via its C-terminal targeting domain, stimulating BiP's ATPase activity and promotes the binding of BiP SBD to Ire1 LD [34]. The recruitment of BiP dissociates ERdj4 from the complex, breaking the dimeric, active Ire1 interface and forces Ire1 into a monomeric, inactive state [34]. The resultant repressive Ire1-BiP complex is maintained by NEF exchanges of ADP to ATP, dissociating BiP from Ire1. During ER stress, ERdj4 and BiP are kept from Ire1 association and repression due to the competitive binding of misfolded proteins, permitting Ire1 dimer formation [34]. This model of BiP/Kar2 reversibly dissociating from Ire1 α /Scire1 was therefore put forth as a driving force regulating Ire1 α /Scire1 activation. Other studies have however experimentally refuted these conclusions. Mutational studies of the Kar2 binding site in the luminal domain of Scire1 show that the loss of the Kar2 binding site does not alter Scire1 activation

and results in the splicing of its downstream target mRNA *HAC1* similar to wild-type cells in the presence of unfolded protein stress [35, 36]. With the current biochemical and structural understanding of Ire1 α /Scire1-BiP/Kar2 interaction, the role of BiP/Kar2 in regulating Ire1 α /Scire1 activity is still relatively unknown. Although it is accepted that BiP/Kar2 reversibly associates to Ire1 α /Scire1, the current model of indirect activation does not explain how BiP/Kar2 dissociation leads to Ire1 α /Scire1 oligomerisation [23, 37, 38].

The direct activation model is based on the crystal structures of yeast and human Ire1 luminal domain. Crystallisation study of the dimerised Scire1 luminal domain was shown to form a ligand binding groove [39]. In particular, the architecture of the ligand binding groove consists of β sheet floors and α helical walls similar to the major histocompatibility complex (MHC) class 1 groove, strongly suggesting its direct ligand binding capacity [39]. Although it was previously reported that the crystal structure of the peptide-binding groove in the luminal domain of human Ire1 α (hIre1 α) was reported to be too narrow to enable peptide binding [23], recent NMR experiments support the notion of the direct activation model of hIre1 α [40]. Conformational changes of the luminal domain between a 'closed' and 'open' state enables peptide binding in the MHC-like groove similar to the yeast ortholog [39, 40]. The direct association of misfolded proteins to the luminal domain of hIre1 α stabilises the open conformation leading to oligomerisation [40]. In addition, BiP/Kar2 and the luminal domain of Ire1 α /Scire1 bind to distinct subsets of proteins, with preferential biases towards specific amino acids, rendering the indirect activation model less likely [24, 40]. Both the dimerised groove of the luminal domain of hIre1 α and Scire1 have an affinity for basic and hydrophobic regions of misfolded proteins, binding them as direct ligands [24, 40]. In addition, it is important to note that BiP/Kar2 are expressed in abundance in the ER lumen while Ire1 α /Scire1 is expressed at significantly lower levels (approximately 100 copies per cell in *S. cerevisiae*) [41]. It is therefore unlikely that BiP/Kar2 binding to substrates must be saturated before Ire1 α /Scire1 can be activated, as Ire1 α /Scire1 dynamically senses ER stress. Since Ire1 α /Scire1 senses and binds a subset of proteins distinct from BiP/Kar2, it is plausible the luminal domain directly senses misfolded proteins despite the sheer abundance of BiP/Kar2 in the ER. Furthermore, in vitro biochemical evidence demonstrated that the luminal domain of Scire1 indeed binds to aggregated substrates [42]. Finally, mutational analysis of residues crucial for peptide binding within Ire1 luminal domain resulted in the loss

of its activity, further supporting the direct activation model [24, 39, 40]. With the evidences described above, we are more inclined towards this mode of sensing and activation by Ire1.

The third and most recent, describes the “allosteric” model of BiP/Kar2 activation of Ire1. In this model, instead of BiP SBD associating to Ire1 α LD (indirect activation model), BiP binds to Ire1 α LD via the NBD and is released upon the binding of misfolded proteins to the BiP/Kar2 SBD [25, 43]. The mechanism of BiP as a direct ER stress sensor, associating to misfolded proteins was recently revealed [44]. The authors showed *in vitro* that BiP shuttles between two cycles: a BiP chaperone cycle and a BiP ER stress sensor cycle depending on its associating partners. In the BiP chaperone cycle, the binding of co-chaperones ERdjs to BiP results in normal chaperone functions. In the BiP ER stress sensor cycle, the association to Ire1 α via its NBD inhibits BiP ATPase stimulation by preventing the binding of ERdjs. This inhibition of ATPase activity prevents BiP from functioning as a Hsp70 chaperone instead acting as an ER stress sensor recognising misfolded proteins resulting in the allosteric release of BiP from Ire1 α . The release of BiP now allows the activation of Ire1 α and the association of co-chaperones allowing it to perform its chaperone functions [44].

Regardless of the activation model, the association of misfolded proteins to the luminal domain triggers conformational changes to the rest of the protein resulting in dimer and higher-order oligomer formation and trans-autophosphorylation [24]. Phosphorylated Ire1 α /Scire1 possesses a ribonuclease activity that cleaves the introns of target mRNAs, such as the pre-mRNA of the transcription factor X-box binding protein 1 (*XBP1*) or orthologous to Atf/Creb1 (*HAC1*) in budding yeast [45, 46] (Figure 1A,B). Importantly, its activation initiates a spliceosome-independent mRNA splicing event. Ire1 α unconventionally splices *XBP1* (*XBP1s*) at a dual stem loop to remove a 26-nucleotide segment [47]. The remaining fragments are subsequently ligated by the tRNA splicing ligase RtcB, generating a product with a frameshift that allows *XBP1s* to be translated into a transcription factor to activate downstream cytoprotective genes [47, 48]. The unconventional splicing of *HAC1* is initiated by the docking of its 3' UTR to the cytosolic domain of oligomerised Scire1 [49]. Only when the intron is removed by Scire1 upon ER stress activation, will *HAC1* spliced (*HAC1^s*) mRNA be translated and ligated by the tRNA ligase Rlg1 to function as a potent bZIP transcription factor (Figure 1B). Hac1 recognises the *cis*-acting element *UPRE* in the promoter regions and initiates a transcriptional

programme, which consists of approximately 381 genes that are involved in many cellular processes, including the phospholipid metabolic pathway, ER-associated protein degradation (ERAD), protein translocation across the ER membrane, vesicular trafficking, glycosylation, cell wall biogenesis and vacuolar protein sorting [50]. Genes regulated by both XBP1 and Hac1 collectively resolve ER stress by increasing the volume of the ER through membrane expansion [51, 52], enhanced protein folding and degradation pathways, as well as translational repression [53].

To further reduce protein load on the ER, Ire1 α cleaves ER-localised mRNAs by an additional mechanism termed regulated Ire1-dependent decay of mRNA (RIDD) (Figure 1A). Even though ScIre1 does not possess RIDD activity, its counterpart in *S. pombe* exclusively relies on RIDD to decrease mRNA load on the ER, due to the lack of *HAC1* or *XBP1* to initiate transcriptional responses (Figure 1C). A group of 471 mRNAs were identified as substrates of Splre1. They are cleaved at specific UG/C sites in the coding region and the 3' UTR mRNA region [54, 55]. Most of these RIDD mRNAs encode for secretory pathway proteins containing either a signal sequence or a transmembrane domain. Importantly, a subset of mRNAs cleaved by Splre1 is stabilized instead of being degraded, contributing to ER homeostasis. In metazoans, because Ire1 α has dual activities for both unconventional splicing and RIDD, *XBP1* and RIDD substrates can be distinguished by the oligomerisation state of Ire1 α [56]. While splicing of *XBP1* or *HAC1* requires oligomerised Ire1 α /ScIre1, RIDD activity is only dependent on monomeric Ire1 α [56, 57].

The other two UPR branches have also been well characterised. The major responding strategies of these branches are conceptually similar to Ire1 α in that they also reduce ER load and/or induce UPR targets. Like Ire1 α , PERK dimerises and auto-phosphorylates upon the sensing of unfolded proteins by its luminal domain [25] (Figure 1A). Once activated, PERK phosphorylates the eukaryotic translation initiation factor 2 α (eIF2 α), resulting in the temporary attenuation of overall protein translation, except for the mRNA of the transcription factor *ATF4* (Figure 1A). ATF4 then activates the transcription of specific UPR target genes, such as CCAAT-enhancer-binding protein homologous protein (CHOP) and the growth arrest and DNA damage-inducible protein (GADD34) (Figure 1A). CHOP is a transcription factor that triggers cell death by inducing genes involved in apoptosis in the presence of persistent

stress [58]. GADD34 on the other hand counteracts the PERK pathway by dephosphorylating eIF2 α to reverse translation attenuation and re-establish the balance [59].

ATF6 is activated by regulated intramembrane proteolysis. Upon ER stress, ATF6 is packed into coat protein complex II (COPII) vesicles and transported to the Golgi apparatus [60]. Cleavage of the luminal and transmembrane domain of ATF6 occurs by membrane-bound transcription factor site-1 protease and site-2 protease in the Golgi, after which the N-terminal cytosolic fragment ATF6(N) localises into the nucleus to activate UPR target genes (Figure 1A).

1.2 The emerging roles of the UPR in metabolic syndromes

In recent years, great efforts have been undertaken to better understand the roles of cellular stress responses in metabolic syndromes. Surprisingly, the UPR, initially perceived as a means of counteracting protein misfolding, emerged as a key pathway [61]. In hindsight, the link should have been anticipated since the disruption of lipid homeostasis compromises ER function. As a stress-response pathway that senses ER stress and activates a transcription programme to control lipid biosynthetic genes, the UPR is ideally positioned to modulate any lipid disequilibrium. Indeed, either prolonged dysregulation of lipid synthesis, or failure of UPR activation to resolve insults, disrupt the homeostatic ER environment and leads to disease development.

The initial clue to such a role can be traced back prior to the inception of the UPR field. Ire1 was first identified in *S. cerevisiae* as a protein required for synthesis of inositol, the essential building block for phospholipids [62] (Figure 3A), before it was later on re-discovered as the master regulator of the UPR [3-5]. Other evidence shows that induction of the UPR by ER-stress inducer drugs upregulates a series of lipid metabolic genes [50]. Reciprocally, deletion of genes involved in lipid biosynthesis triggers the activation of the UPR [63-65]. These early insights appeared to be somewhat indirect until recent work provided solid evidence to justify the UPR as a lipid regulator [66, 67].

In mouse liver, Ire1 α is also required for the assembly and secretion of very low-density lipoprotein (VLDL) and its secretion in hepatocytes [68]. Upon *IRE1 α* deletion, excessive amounts of triglycerides accumulate in hepatocytes regardless of fasting or high fructose diet while TAG biogenesis is

unaffected. Downstream of Ire1 α , XBP1 is required for normal fatty acid synthesis, and its deletion results in hypocholesterolemia and hypotriglyceridemia in addition to activating the regulated Ire1-dependent decay (RIDD) activity of Ire1 α [69]. As targets of RIDD include mRNAs that encode proteins in lipogenesis and lipoprotein metabolism, diminishing the RIDD activity by knocking-down *IRE1 α* reverses hypolipidemia in *XBP1*-deficient mice [70]. In addition, XBP1 also serves as a regulator for lipogenesis and responds to high dietary carbohydrate by inducing genes that control fatty acid and cholesterol synthesis [69]. Furthermore, *XBP1*-deficient obese mice develop insulin resistance, suggesting that XBP1 has a role in protecting cells from metabolic disorders through the activation of UPR genes [71]. Unexpectedly, XBP1 was shown to directly interact with the forkhead box O1 (FoxO1) transcription factor and target it for proteasomal degradation [72]. FoxO1 negatively regulates hepatic glucose metabolism by modulating expression of and promoting the degradation of hepatic gluconeogenic enzymes [73]. This novel mechanism indicates that XBP1 has a dual role in modulating serum glucose levels, which is independent of the cytoprotective effects it exerts on the ER-folding capacity. These findings clearly undermine the protective roles of the Ire1 branch in maintaining hepatic lipid metabolism.

The PERK branch of the UPR is also involved in the metabolic response. CHOP, a downstream target of PERK, has been shown to activate the NF- κ B pathway from saturated free fatty acid (SFA)-induced lipotoxicity [74], suggesting a role in the pathogenesis of NASH and hepatic inflammation (Figure 1A). CHOP-induced NF- κ B promotes the secretion of proinflammatory cytokines, contributing to hepatic inflammation and the severity of NASH. SFA-induced lipotoxicity in apoptotic β -cells similarly induces the PERK/CHOP axes and plays a role in development of NASH and type II diabetes [75]. Rebalancing the PERK pathway by enforced expression of GADD34 to dephosphorylate hepatic Eif2 α enhances glucose tolerance and attenuates hepatosteatosis in mice fed on a high-fat diet [76] (Figure 1A).

Although it is the least studied UPR activation pathway, ATF6 might also contribute to lipid homeostasis as it can be activated upon lipid bilayer stress [75, 77]. *ATF6* ablated in mice, subjected to pharmacological ER stress, leads to hepatosteatosis due to the dysregulation of lipid metabolism, highlighting ATF6 critical role in maintaining phospholipid homeostasis [78]. Recently, a novel lipid sensing mechanism of ATF6 was demonstrated. A specific transmembrane domain motif directly

sensing the accumulation of early sphingolipid intermediates dihydrosphingosine (DHS) and dihydroceramide (DHC) independently of its luminal domain, resulted in the preferential activation of lipid biosynthetic genes [79]. This was the very first report of the dual sensing potential of ATF6.

Figure 3. Simplified schematic illustration of the major lipid metabolic pathways. (A) Major phospholipids synthesis pathways. Phosphatidic acid (PA) is the product of two acyl-CoAs incorporated into glycerol 3-phosphate (glycerol 3-P). Converted from PA, diacylglycerol (DAG) is the precursor of phosphatidylethanolamine (PE), phosphatidylcholine (PC), and triacylglycerol (TAG) while cytidine diphosphate choline-DAG (CDP-DAG) is the precursor for phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylglycerol (PG). PI can be polyphosphorylated (PI_x) and PG can be dimerised to cardiolipin (CL). The *de novo* biosynthesis of PC from PE occurs in a three-step methylation reaction through the intermediates monomethyl-PE (MMPE) and dimethyl-PE (DMPE). PS is converted to PE via the *de novo* pathway. (B) Sterol ester biosynthesis pathway. Sterols are biosynthesized from a series of intermediate products including the rate limiting step where 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is reduced into mevalonic acid. (C) Sphingolipid biosynthesis pathway. The condensation of serine to palmitoyl-CoA is the first rate-limiting step leading to ceramide through intermediate products. Ceramide can be further modified to form complex sphingolipids such as sphingosine-1-phosphate (S1P). Several sphingolipids and sterol intermediates are omitted in this figure for simplification. Genes in green and blue refers to mammal and yeast, respectively. CDP-Cho, cytidine diphosphate choline; CDP-ETA, cytidine diphosphate ethanolamine; CoA, coenzyme A; Ino, inositol; IPC, inositolphosphorylceramide; MIPC, mannosylinositolphosphorylceramide; M(IP)₂C, mannosyldinositolphosphorylceramide, PGP, phosphatidylglycerolphosphate. Figure modified from [22].

1.3 ER membrane composition and UPR induction

The homeostatic environment of the ER relies on a fine-tuned balance between hundreds of membrane lipids and is essential in maintaining the stability of numerous membrane proteins and the proper folding equilibrium within the organelle [65, 80]. Aberrancies in lipid biosynthesis cause ER stress and triggers the activation of the UPR [64, 81] (Figure 3). Here, both Ire1 α and PERK can respond to lipid bilayer stress independent from sensing protein misfolding through their luminal domains. The transmembrane and proximal domains of Ire1 α and PERK are positioned to monitor changes within the lipid bilayer. Importantly, Ire1 α and PERK mutants that lack their luminal domains (Δ LD-Ire1 α and Δ LD-PERK) and are thus unable to respond to the accumulation of unfolded proteins, still retained their activity in initiating the UPR, *i.e.* *XBP1* splicing and eIF2 α phosphorylation, respectively, upon membrane saturation-induced LBS [66] (Figure 1A).

Similarly, the Δ III Ire1 in *S. cerevisiae* activated the UPR independent of subregion III of the core stress sensing domain in the luminal domain, responsible for misfolded protein dependent activation of the UPR, when starved of inositol. Further work suggests that the N-terminal transmembrane domain of ScIre1 functions as an amphipathic helix to detect any biophysical changes at the ER membrane [67]. Based on *in vivo*, *in vitro* and molecular dynamics simulation evidences, Halbleib *et al.* proposed that the local membrane compression caused by the juxta-membrane amphipathic helix of ScIre1 increases during LBS [67]. The increased energetic cost for membrane compression by each Ire1 molecule can be minimised by the formation of transient dimeric configurations, stabilised by the membrane environment [67]. Reconstitution of this N-terminal amphipathic helix and the transmembrane helix into liposomes revealed a stabilising tilted orientation assumed by the transmembrane helix in response to lipid bilayer stress conditions (increased lipid order) that allows for oligomerisation and Ire1 activation. PERK likely senses lipid disequilibrium through a similar mechanism given it has a high similarity to the transmembrane domain Ire1 α . Indeed, the transmembrane domain of Ire1 α and PERK can be swapped without compromising the lipid-sensing activity [66]. New evidence in Ire1 α however discovered that there are crucial structural features within Ire1 α transmembrane helix that influences its responsiveness to membrane saturation [82]. Although the amphipathic helix is conserved from yeast to humans, the mutation of residues controlling the sensing abilities of the amphipathic helix in Ire1 α did not inactivate the protein. Instead, a tryptophan and serine residue within the α -helix of Ire1 α was necessary for the

support and stability of dimer formation during palmitate exposure [83]. This evidence further emphasises the role of the transmembrane domain as a lipid sensor.

Saturated fatty acid palmitic acid has been extensively added to growth media to induce LBS in cell culture (Figure 4). This excessive intake of SFA changes the metabolic flux of phospholipid synthesis and leads to an increased amount of saturated lipid species at the ER membrane [84]. High degree of fatty acid saturation at the biological membrane affects membrane fluidity by promoting stiffening of the membrane [85-87]. It is likely that membrane-protein interaction dynamics are altered by the decrease in membrane fluidity, producing a signal that is eventually received by the UPR sensors and amplified in the initiation of the UPR programmes [88]. SFA-induced UPR in INS-1E β -cells can be counteracted by supplementing growth media with unsaturated oleic acids, bringing back the delicate balance between saturated and unsaturated FAs [89] (Figure 4). Inhibiting the conversion of SFA to monounsaturated FA by targeting stearoyl-CoA desaturase genetically or pharmacologically leads to similar LBS-induced UPR as supplementing growth media with SFA [66, 90]. The mechanisms underlying these observations seem to be conserved throughout evolution. In *S. cerevisiae*, increases in membrane saturation owing to deletion of 5-aminolevulinate synthase *HEM1* or desaturase *OLE1* results in UPR activation [91]. Here, supplementation of monounsaturated fatty acids myristoleic acid (C14:1) and oleic acid (C18:1) to correct the unbalanced ratio between saturated and unsaturated phospholipids relieved ER stress, whereas SFA palmitic acid (C16:0) supplementation to *hem1* Δ cells further exacerbates the UPR activation [91] (Figure 3). Loss of *HEM1* leads to a defect in ergosterol and unsaturated FA synthesis [92, 93] (Figure 3). Additionally, a yeast based fatty acid screening assay demonstrated that the addition of the long chain monounsaturated oleic acid is particularly effective to shut down UPR activation and overcome ER stress in *hem1* Δ cells [94]. Surprisingly, some long chain polyunsaturated fatty acids that are absent in *S. cerevisiae* significantly improved the growth of *hem1* Δ cells. LBS-induced UPR can also be triggered by the deletion of ubiquitin-like (UBX)-domain-containing protein (*UBX2*), an important component of the ERAD pathway. The absence of *UBX2* triggers the accumulation of saturated fatty acid as the transcription control of the FA desaturase *OLE1* is impaired [95]. The upregulation of *OLE1* is mediated through multicopy suppressor of Gam1 (*MGA2*) that activated by the ERAD machinery. Interestingly, Mga2 was recently shown to be an important regulator of membrane fluidity by acting as a sensor of membrane lipid saturation and controlling unsaturated fatty acid synthesis by the desaturase Ole1 [96]. These findings suggest that membrane properties,

such as those defined by the ratio between saturated and unsaturated phospholipids and acyl chain length, are constantly monitored by the UPR.

The ER membrane also contains sphingolipids, such as ceramide, sphingosine and sphingosine 1-phosphate (S1P), which are, however, only in low abundance compared to phospholipids (Figure 3C and 4). It is evident that sphingolipids are necessary to maintain lipid homeostasis and prevent ER stress [97]. The first rate-limiting step in sphingolipid synthesis is carried out by the serine palmitoyltransferase complex (Spt), which is negatively regulated by the evolutionarily conserved *ORM1* and *ORM2* (Figure 3C) [98]. Accordingly, deletion of *ORM1* or *ORM2* leads to lipid-mediated UPR activation [63, 99, 100]. Aberrant sphingolipid composition has also been found to be involved in the pathogenesis of obesity and insulin resistance. Indeed, ceramide accumulates in mice adipose and muscle tissues upon exposure to high fat diet [101]. Furthermore, it has been shown that the accumulation of C16 ceramide upon UPR activation leads to cell death through an induction of pro-apoptotic factor Bax leading to cytochrome c release and mitochondrial apoptosis [102-104]. In obese mice, cell death can be avoided by overexpression of sphingosine kinase 1 (SphK), which promotes the conversion of ceramide to S1P [105] (Figure 3C). S1P functions by attenuating the proinflammatory JNK pathway, thereby ameliorating high fat diet induced insulin sensitivity partly by reducing inflammation of muscle cells. Importantly, it should be noted S1P-phosphohydrolase 1 (Spp1), a phosphatase of S1P plays a role in maintaining ER homeostasis [106] (Figure 3C). S1P accumulation upon Spp1 deletion stimulates autophagy and ER stress, suggesting that the intricate regulatory mechanisms that are involved in sphingolipid metabolism are also required to maintain ER homeostasis. Only recently was the mechanism of ATF6 lipid sensing reported [79]. Independent of its luminal domain, a transmembrane domain motif of ATF6 senses and binds the specific sphingolipid precursors dihydrosphingosine (DHS) and dihydroceramide (DHC) for its activation [79]. While DHC-activated ATF6 was still packaged into COPII vesicles and cleaved by S1P and S2P, distinct differences were observed between DHC activated ATF6 versus DTT-induced proteotoxic stress [79]. One such difference was the lack of BiP dissociation from ATF6 in the presence of DHC [79]. Interestingly, the transcriptional response of DHS/DHC induced ATF6 activation differed from that of proteotoxic stress, resulting in the preferential upregulation of ER lipid biosynthetic genes and metabolic genes over chaperone genes [79].

Another lipid feature important for ER homeostasis is the optimal ratio of phospholipids with different head groups [107, 108]. The ER membrane is composed predominantly of approximately 40% phosphatidylcholine (PC) and 20% phosphatidylethanolamine (PE) in yeast and present in higher amounts with 55% PC and 30% PE in mammals while phosphatidylinositol and phosphatidylserine are of lower abundance [109, 110]. A prolonged imbalance in the ratio between PC and PE has been shown to activate the UPR and cause NASH [111]. This can be explained by the distinct biophysical properties of these two classes: PC resembles a cylindrical shape allowing for better packing and bilayer forming propensity, whereas PE is conically shaped lacking a bilayer forming ability due to packing rigidity [112, 113] (Figure 4 and 5A,B). The non-bilayer forming propensity of PE depends on the acyl chain composition with double bonds [114]. Changes to PE levels affects the trans bilayer distribution of different PE species, which are defined by intrinsic membrane curvature and cholesterol content [112, 115] (Figure 5B). The distribution of PE species amongst the membrane is critical for the function of membrane processes potentially affecting UPR membrane protein functions. It was shown that the depletion of PC by *cho2Δopi3Δ* results in the shortening of the acyl chains and increase in the degree of saturation of PE [116]. The resultant change in membrane integrity and rigidity create imbalances between PC and PE levels that is alternatively sensed by the UPR sensors [111]. Indeed, the deletion of the major yeast ER phospholipid PC biosynthetic genes *CHO2* and *OPI3* triggered hyperactivation of the UPR, which buffers the otherwise lethal defect this imbalance would have [65]. Mostly expressed in the liver, *PEMT*, the homologue of *OPI3*, is a methyltransferase required for *de novo* synthesis of PC from PE (Figure 3A). *PEMT* deletion in mice (*Pemt*^{-/-}) causes liver failure after three days of choline-deficient diet [117]. The reduction of the PC/PE molar ratio in liver tissues of both *Pemt*^{-/-} mice and human patients resulted in the loss of membrane integrity due to the disproportional increase of the non-lamellar lipid bilayer forming PE, suggesting an increase in intrinsic membrane curvature [118, 119] (Figure 5B). In addition to membrane integrity, aspects of membrane dynamics, including lipid-lipid and lipid-protein interactions, are also compromised by the loss of PC [120]. In obese mice, the increased PC/PE ratio impairs the function of sarco/endoplasmic reticulum calcium ATPase (SERCA) and activates the UPR [121]. The impairment of the SERCA pump affects Ca²⁺ levels within the ER, affecting chaperone functions and protein folding consequently activating the UPR. Furthermore, an imbalanced PC/PE ratio can also result in the aberrant activation of downstream signalling pathway and thus

eventually might lead to disease manifestation. For example, the JNK signalling pathway is hyperactivated in cells with a dysregulated PC/PE ratio; this suppresses the insulin signalling pathway through phosphorylation of insulin receptor substrate-1 (IRS-1), which gives rise to insulin resistance, a hallmark of type II diabetes [71]. In addition, dysregulation of the PE biosynthetic pathway in hepatic steatosis was found to result in abnormal accumulation of neutral lipids, phospholipid remodelling and upregulation of lipogenic transcription factors, which all contributed to the development of the disease [122, 123]. Taken together, aberrant changes in lipid bilayer composition are sensed by the UPR and these have been implicated in the onset of diseases, particularly obesity and diabetes.

Figure 4. Lipid classes and representatives. Structures in red and black highlight polar and nonpolar regions, respectively. Representative phospholipids are as follow: phosphatidylcholine (PC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; phosphatidylethanolamine (PE), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine; phosphatidylserine (PS), 1,2-dipalmitoyl-sn-glycero-3-phosphoserine, and phosphatidylinositol (PI), 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'-myo-inositol). Sphingomyelin is represented by N-palmitoyl-D-erythro-sphingosylphosphorylcholine and sterols as cholesterol. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Figure 4 is not representative of acyl chain length and saturation of phospholipids found physiologically within the cell. The emphasis of this figure is on the size of the headgroup of each phospholipid which is then illustrated in Figure 5. Yeast membranes consists of mostly di- and mono-unsaturated phospholipids of acyl chain length C16:1, C18:1, C16:0 and C18:0 [114, 124]. Figure modified from [22].

Figure 5. Lipid shapes and physical properties. Properties of a lipid bilayer affect its shape and fluidity. (A) Molecular shapes of lipids are defined by their polar headgroup (red) and fatty acid saturation states (grey). PC (phosphatidylcholine), PI (phosphatidylinositol) and PS (phosphatidylserine) form cylindrical-like shapes and PE resembles a cone, whereas phospholipids containing one instead of two acyl chain (lyso-PC) exhibit an inverted conical geometry. (B) Packaging of cylindrical lipids results in a planar-shaped lipid bilayer, whereas inverted-cone lipids and cylinders will be sorted into positively and negatively curved lipid bilayers, respectively. (C) Localized properties of lipids can result in a thickening or thinning of the lipid bilayer. For instance, clustering of cholesterol and glycosphingolipids will form a thicker microdomain, referred as lipid raft-like domains. Conversely, the concentration of phospholipids with short acyl chains will reduce the thickness of the lipid bilayer. Reproduced with permission from [22].

1.4 Aim

Given the intimate relationship between lipid dysregulation and the UPR, how the UPR transmembrane protein Scire1 senses changes in lipid composition of the ER membrane remains unknown. Here, we make use of an experimental model of NASH in *Saccharomyces cerevisiae* by the genetic knockout of the homolog of PEMT, the methyltransferase Opi3. In the $\Delta opi3$ mutant, PC content decreases 173 folds, being effectively absent, therefore establishing the conditions of lipid bilayer stress (LBS) in the ER membrane [65]. The truncated form of Ire1, Ire1 Δ LD (also referred to as in short as I Δ LD in this thesis), lacking its luminal domain and therefore its protein stress sensing ability was constructed and expressed in lipid bilayer stress yeast strains to study the activation mechanism of Ire1 in response to

LBS (Figure 6). The advantage of *S. cerevisiae* as a model organism is the single UPR branch present that eliminates the possibility of crosstalk of the two other UPR pathways.

In yeast, lipid dependent activation of the UPR has been demonstrated through several means, such as the loading of yeast with saturated fatty acids and sterol, by deletion of regulators of sphingolipid synthesis Orm1 and Orm2 and inositol depletion [91, 99, 125]. Inositol depletion has been commonly used as a form of lipid bilayer stress model since it is an essential precursor of the membrane phospholipid phosphatidylinositol (Figure 3), the lack thereof causes UPR activation [126]. In a previous study, to determine other stress stimuli that activate the UPR, a Δ III Ire1 mutant, truncated at position 243-272 in the luminal domain of yeast Ire1, was constructed [64] (Figure 7). Removal of the Δ III subregion of Ire1 renders a delayed response to UPR^{PT}, with peak UPR activation 5 hours post DTT treatment instead of 30 min in WT cells post DTT treatment. This enables Δ III Ire1 to be used to study other stress stimuli; gene deletions that activate the UPR independently its misfolded protein stress sensing domain such as membrane aberrations by inositol depletion. While only a collection of 18 genes have been studied for the effects of their deletion of the activation of the UPR during lipid bilayer stress by the Δ III Ire1, we sought to seek a global picture of the biological processes that are maintaining the UPR during lipid bilayer stress through a high content yeast deletion library screen using the Ire1 Δ LD strain. We identified an overlap of 181 genes whose deletion caused an upregulation of the UPR in Ire1 and Ire1 Δ LD cells and discovered the importance of genes associated to protein folding, ER associated degradation (ERAD), vesicular trafficking and lipid metabolism required for ER membrane integrity during lipid bilayer stress. By genetic knockdown and biochemical analysis, we show here that in *S. cerevisiae*, the luminal domain of Ire1 is not required for UPR activation during LBS. We hypothesize that the domain in direct contact with the lipid bilayer is the domain responsible for sensing changes to membrane lipid properties. While transmembrane domain residues within Ire1 α have been identified to be important for lipid bilayer stress activated UPR (UPR^{LBS}), there is no evidence of regulatory residues within the transmembrane domain of ScIre1. This led us to explore the role of transmembrane domain of ScIre1, as a sensor during lipid bilayer stress, in a metabolic context. We identify an arginine residue at position 537 within the transmembrane domain driving the activation of Ire1 under LBS. We also describe the oligomerisation state of Ire1 and Ire1 Δ LD between misfolded protein activated UPR (UPR^{PT}) and UPR^{LBS}.

DNA microarray analysis revealed the transcriptional upregulation of six novel UPRE-containing genes during UPR^{LBS}. ChIP-qPCR analysis was performed to determine Hac1 association to the UPRE-containing promoter of these six genes to determine if they are potential UPR target genes. We confirmed the association of Hac1 to three genes: *PUT1*, *PBI2* and *PIR3*, suggesting their unidentified role in the homeostatic response mounted during UPR^{LBS} and the divergent transcriptional response adopted by cells under different stresses (UPR^{PT} versus UPR^{LBS}). In summary, we attempt to dissect the activation mechanism of Scire1 and the form of adaptive biological processes and divergent transcriptional programmes initiated during UPR^{LBS}, commonly observed in liver disease. Our data suggests a unique role of Ire1 as a sensor for detecting changes in membrane properties of the ER, in addition to its role of misfolded protein sensing.

Figure 6. Characterising UPR^{LBS} by Ire1 Δ LD. Full length Ire1 can be activated by both the association of unfolded proteins to the luminal domain and by changes to membrane properties of the ER while the Ire1 Δ LD can only be activated directly by lipid bilayer stress.

ER

Figure 7. The ΔIII Ire1 mutant is mostly activated by lipid bilayer stress. The luminal domain of yeast Ire1 is classified into five subregions as indicated. Subregion III (amino acids 243-272), the core stress-sensing region (CSSR) was truncated (ΔIII Ire1) to delay its protein stress sensing ability. This enables ΔIII Ire1 to detect other forms of stresses activating the protein. Figure adapted from [64].

Chapter 2. Materials and methods

2.1 Strains construction and genetic recombination methods

S. cerevisiae strains used in this study are listed in Table 1. Strains were prepared using standard transformation protocols. High efficiency transformation protocol was performed using the lithium acetate method as previously described [127]. In brief, cells were grown to 0.6-0.8 OD₆₀₀/ml in 10 ml of selective media. Cells were pelleted at 1000 x g, 5 min, 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 added to PLATE mix (40% PEG-3350, 100 mM LiOAc, 10 mM Tris-HCl pH 7.4, 1 mM EDTA), ssDNA and PCR product or plasmid DNA and incubated at 30 °C, 1 hour. DMSO was then added to the transformation mixture and heat shocked at 42 °C for 22 min. Cells were recovered in rich YPD media before plated onto the appropriate selection plates. Successful transformants that grew after incubation at 30 °C for 48-72 hours were verified by genotyping.

Table 1. Yeast strains used in this study

Strain	Genotype	Background	Plasmid(s)	Reference
W303a	<i>leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15</i>			[4]
BY4741	<i>his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>			[128]
YGT0012	<i>MATa ire1::trp</i>	W303		
YGT0106	<i>MATa ire1::G418</i>	W303		This study
YGT0125	<i>MATa ire1::G418, opi3::G418</i>	W303		
YGT0268	<i>MATa, ire1::G418, opi3::G418</i>	W303	pGT0201	This study
YGT0269	<i>MATa, ire1::G418, opi3::G418</i>	W303	pGT0330	This study
YGT0270	<i>MATa, ire1::G418</i>	W303	pGT0201	This study
YGT0271	<i>MATa, ire1::G418</i>	W303	pGT0001	This study
YGT0272	<i>MATa, ire1::G418</i>	W303	pGT0330	This study
YGT0276	<i>MATa, ire1::G418, opi3::G418</i>	W303	pGT0012, pGT0330	This study
YGT0278	<i>MATa, ire1::G418</i>	W303	pGT0001, pGT0012	This study
YGT0279	<i>MATa, ire1::G418</i>	W303	pGT0012, pGT0330	This study
YGT0281	<i>MATa, ire1::G418, opi3::G418</i>	W303	pGT0001	This study
YGT0285	<i>MATa, ire1::G418</i>	W303	pGT0223	This study

Strain	Genotype	Background	Plasmid(s)	Reference
YGT0286	<i>MATa, ire1::G418</i>	W303	pGT0225	This study
YGT0287	<i>MATa, ire1::G418, opi3::G418</i>	W303	pGT0223	This study
YGT0288	<i>MATa, ire1::G418, opi3::G418</i>	W303	pGT0225	This study
YGT0551	<i>MATa, ire1::G418, opi3::G418</i>	W303	pGT0289, pGT0330	This study
YGT0554	<i>MATa, ire1::G418</i>	W303	pGT0001, pGT0289	This study
YGT0700	<i>MATa, ire1::trp</i>	W303	pGT0001	This study
YGT0701	<i>MATa, ire1::trp</i>	W303	pGT0330	This study
YGT0702	<i>MATa, ire1::trp</i>	W303	pGT0201	This study
YGT0704	<i>MATa, ire1::trp, opi3::G418</i>	W303	pGT0330	This study
YGT0705	<i>MATa, ire1::trp, opi3::G418</i>	W303	pGT0201	This study
YGT0719	<i>MATa, ire1::G418, opi3::G418</i>	W303	pGT0330, pGT0334	This study
YGT0727	<i>MATa, ire1::KanMX, ura3::IRE1-3XFLAG, his3::HA-HAC1-HIS3</i>	W303		[129]
YGT0804	<i>MATa, ire1::G418</i>	W303	pGT0003, pGT0004, pGT0012, pGT0330	This study
YGT0805	<i>MATa, ire1::G418, opi3::G418</i>	W303	pGT0003, pGT0004, pGT0012, pGT0330	This study
YGT0825	<i>MATa, ire1::G418</i>	W303	pGT0001, pGT0002, pGT0004, pGT0012	This study
YGT0826	<i>MATa, ire1::G418, opi3::G418</i>	W303	pGT0004, pGT0012, pGT0330, pGT0334	This study
YGT0827	<i>MATa, ire1::G418</i>	W303	pGT0004, pGT0012, pGT0330, pGT0334	This study
YGT0828	<i>MATa, ire1::G418</i>	W303	pGT0003, pGT0012, pGT0330, pGT0421	This study
YGT0829	<i>MATa, ire1::G418, opi3::G418</i>	W303	pGT0004, pGT0012, pGT0330, pGT0421	This study
YGT1005	<i>MATa, ire1::G418</i>	W303	pGT0003, pGT0012, pGT0201, pGT0421	This study
YGT1006	<i>MATa, ire1::G418, opi3::G418</i>	W303	pGT0004, pGT0012, pGT0201, pGT0421	This study
YGT1118	<i>MATa, ire1::trp</i>	W303	pGT0442	This study
YGT1119	<i>MATa, ire1::trp</i>	W303	pGT0443	This study
YGT1120	<i>MATa, ire1::trp, opi3::G418</i>	W303	pGT0442	This study
YGT1121	<i>MATa, ire1::trp, opi3::G418</i>	W303	pGT0443	This study
YGT1150	<i>MATa, ire1::G418</i>	W303	pGT0001, pGT0448	This study
YGT1151	<i>MATa, ire1::G418</i>	W303	pGT0330, pGT0448	This study

Strain	Genotype	Background	Plasmid(s)	Reference
YGT1152	<i>MATa, ire1::G418</i>	W303	pGT0201, pGT0448	This study
YGT1153	<i>MATa, ire1::G418, opi3::G418</i>	W303	pGT0330, pGT0448	This study
YGT1154	<i>MATa, ire1::G418, opi3::G418</i>	W303	pGT0201, pGT0448	This study
YGT1202	<i>MATa, ire1::ire1ΔLD-HgX UPRE-GFP::URA TEF2-cherry::Met his3Δ1 leu2Δ0 lys2+ met15Δ0 ura3Δ0 can1Δ::STE2pr-sp HIS5 lyp1Δ::STE3pr-LEU2 cyh2</i>	BY4741		[130]
YGT1228	<i>MATa, ire1::ire1-HgX UPRE-GFP::URA TEF2-cherry::Met his3Δ1 leu2Δ0 lys2+ met15Δ0 ura3Δ0 can1Δ::STE2pr-sp HIS5 lyp1Δ::STE3pr-LEU2 cyh2</i>	BY4741		[130]
YGT1258	<i>MATa, ire1::trp</i>	W303	pGT0557	This study
YGT1259	<i>MATa, ire1::trp</i>	W303	pGT0558	This study
YGT1260	<i>MATa, ire1::trp, opi3::G418</i>	W303	pGT0557	This study
YGT1261	<i>MATa, ire1::trp, opi3::G418</i>	W303	pGT0558	This study

2.2 Antibodies used in this study

Anti-HA mouse monoclonal antibodies HA.11 (Covance, Princeton, NJ, catalog #MMS-101R-1000), anti-Flag mouse M2 monoclonal antibody (Sigma-Aldrich, St. Louis, MO, catalog #F-1804-1MG), Anti-Kar2 rabbit polyclonal antibody, anti-CPY rabbit polyclonal antibodies, and anti-Sec61 rabbit polyclonal antibody were gifts from Davis Ng (Temasek Life Sciences Laboratories, Singapore). Secondary antibodies goat anti-mouse IgG-DyLight 488 (Thermo Fisher, Waltham, MA, catalog #35503), goat anti-rabbit IgG DyLight 550 (Thermo Fisher, Waltham, MA, catalog #84541), goat anti-mouse IgG-IRDye 800 (LI-COR Biosciences, Lincoln, NE, catalog #926-32210) and goat anti-rabbit IgG-IRDye 680 (LI-COR Biosciences, Lincoln, NE, catalog #926-68021) were commercially purchased.

2.3 Plasmids and primers used in this study

Plasmids and oligonucleotides used in this study are listed in Table 2 and 3, respectively. Plasmids were constructed either by restriction or Gibson cloning. All coding sequences of plasmid constructs used in this study were fully sequenced. The plasmid pGT0012 containing the unfolded protein response element (*UPRE*) fused to *LacZ* in pRS314 was a gift from Peter Walter and constructed as

previously described [5]. The plasmid pGT0227 containing the heat shock element (*HSE*) fused to *LacZ* in a 2 μ URA plasmid was a gift from Kevin Morano and constructed as previously described [131]. The plasmid pGT0421 containing HA tagged *HAC1* in pMR366 was constructed as previously described [132]. The plasmid pGT0330 containing endogenously *IRE1* was generated as previously described [133]. The plasmid pGT0201 containing *IRE1 Δ LD* was generated by amplifying the endogenous promoter and signal sequence (fragment 1) and transmembrane cytosolic domain (fragment 2) with primer pairs GTO275-276 and GTO277-GTO278 respectively, from template WT genomic DNA (WT gDNA). The fragments were further digested with *XhoI* and *PstI* (fragment 1) and *PstI* and *NotI* (fragment 2) before ligation into a pRS313 *XhoI* and *NotI* linearised plasmid. The plasmids pGT0223 and pGT0225 were generated by amplification using primer pair HWO15-16 and ligated into pSW234. pGT0285 was generated by amplifying the luminal domain with primers B29 containing a *BamHI* cut site and B30 that contain HA-HDEL-*NcoI* overhang sequences followed by ligation into *BamHI/NcoI* linearised pGT0223. The *PGK1* promoter was amplified from pGT0121 with primer pair B36-37 and subsequently digested with *NotI* and *BamHI* and ligated with *BamHI/NotI* linearised pGT0285 to make pGT0289. The plasmids BGT0261 and BGT0262 were generated by the ligation of *NcoI/NotI* digested pGT0101 into *NcoI/NotI* digested pGT0223 and pGT0225. pGT0101 was generated as previously described [134]. pGT0442 and pGT0443 was generated from pGT0261 and pGT0262 respectively by Phusion site-directed mutagenesis using the primer pair HN107-108 as previously described [135]. pGT0448 was a gift from Madhusudan Dey and constructed as previously described [136]. pGT0557 and pGT0558 were generated from pGT0330 and pGT0201 respectively by Phusion site-directed mutagenesis using the primer pair HN107-108. The split venus constructs pGT0544 and pGT0546 were generated by Gibson assembly to join HA-VN173 (synthesized by GBlock) with HN177-178 linearised pGT0330 and pGT0201. The split venus constructs pGT0545 and pGT0547 were generated by Gibson assembly to join FLAG-VC155 (synthesized by GBlock) with HN177-178 linearised pGT0059 and pGT0435. The plasmid pGT0059 containing endogenously expressed *IRE1* was generated by amplification with primer pair HN5F-HN116 from template WT gDNA and Gibson assembled into *SmaI* linearised pGT0003. The plasmids pGT0498 and pGT0564 are gifts from Maya Schuldiner as previously described [63].

Table 2. Plasmids used in this study

Plasmid	Encoded protein	Promoter	Vector	Source
pGT0001	Empty		pRS313	[137]
pGT0003	Empty		pRS315	[137]
pGT0004	Empty		pRS316	[137]
pGT0012	β -galactosidase	<i>UPRE-CYC1</i>	pRS314	[5]
pGT0059	Ire1	<i>IRE1</i>	pRS315	This study
pGT0101	Opi3-FLAG	<i>OPI3</i>	pRS315	[134]
pGT0121	Empty	<i>PGK1</i>	pRS313	This study
pGT0201	Ire1 Δ LD	<i>IRE1</i>	pRS313	This study
pGT0223	Ire1	<i>PRC1</i>	pRS315	This study
pGT0225	Ire1 Δ LD	<i>PRC1</i>	pRS315	This study
pGT0227	β -galactosidase	<i>HSE-CYC1</i>	2 μ	[131]
pGT0261	Ire1-FLAG	<i>PRC1</i>	pRS313	This study
pGT0262	Ire1 Δ LD-FLAG	<i>PRC1</i>	pRS313	This study
pGT0285	Ire1 LD-HA	<i>PRC1</i>	pRS315	This study
pGT0289	Ire1 LD-HA	<i>PGK1</i>	pRS315	This study
pGT0330	Ire1	<i>IRE1</i>	pRS313	This study
pGT0421	Kar2	<i>TDH3</i>	pMR366	[65]
pGT0435	Ire1 Δ LD	<i>IRE1</i>	pRS315	This study
pGT0442	Ire1(R537Q)-FLAG	<i>PRC1</i>	pRS313	This study
pGT0443	Ire1 Δ LD(R537Q)-FLAG	<i>PRC1</i>	pRS313	This study
pGT0448	3X-HA-Hac1	<i>HAC1</i>	pRS316	[136]
pGT0498	GFP	<i>UPRE-CYC1</i>	pRS306	[63]
pGT0544	Ire1-HA-VN	<i>IRE1</i>	pRS313	This study
pGT0545	Ire1-FLAG-VC	<i>IRE1</i>	pRS315	This study
pGT0546	Ire1 Δ LD-HA-VN	<i>IRE1</i>	pRS313	This study
pGT0547	Ire1 Δ LD-FLAG-VC	<i>IRE1</i>	pRS315	This study
pGT0557	Ire1(R537Q)	<i>IRE1</i>	pRS313	This study
pGT0558	Ire1 Δ LD(R537Q)	<i>IRE1</i>	pRS313	This study
pGT0564	mCherry	<i>TEF2</i>	pFA6-natMX6	[63]

Table 3. Oligonucleotides used in this study

Primer	Sequence (5' to 3')
HN5F	CTAGAACTAGTGGATCCCCCGTCTTATCCTTGCCATAAACAGAAGACAACCTTGCAAG
HN46	CTGGAAATTTACCCGGC
HN47	CAGTAACACGTTAATCTTTAGAATTAC
HN56	CAGCCCTTATTTTTTTTCCAAG
HN57	GGGATGCGTGGTATTG
HN58	CACGTACATTGCTGTGG
HN59	AGTGCACGCTCAACT
HN60	CTCCTGATAGACAATGAAATATAACC
HN61	AGTGCTACTAGGCGATG
HN62	CCCTATCTTTTTTTTTTCTCGC
HN63	ATGCAGTGAACTAAGGATAC
HN64	GCAAGATGATGTTCAACCT
HN65	TACTTGGCATATTGCTTAACTC
HN66	GTGAAAAATGCATCCAAAAGA
HN67	TACTACATTCGGAAATGGTTG
HN107	CCAGTCTCTATAATTTGATATACTAGACTTCC
HN108	AGTATTTCTGTTGTTATTTCTCATTTTTTG
HN116	CTTGATATCGAATTCCTGCAGCCCCACCAAAAAAGTCAGTG
HN117	TTAGGCGGCCGGAGCGTAA
HN118	GGGCTGCAGGAATTCGATATCAAGCT
HN119	GACATTTAATTTTATAAATACATATCTCATAAACAGCGACATGGAGGCCAG
HN120	AACTGGAGTAGTATGTCGATGTTGATTATAAATAACGTTCTTAATACTAAC
HN121	GTTTATGAGATATGTATTTATAAAATTAATGTCTGTC
HN122	ATCGAACATCGACATACTACTCC
HN148	ACAAAGAAGTAATGAACTTAAATGCTATTATACAG
HN149	CCGTCCCAAACATTGTCATAGATTC
HN153	CATGTTTCATGCCCCTCTGC
HN177	TGAATACAAAAATTCACGTAAAATTTGATCGTCAC
HN178	TAACATGTTTCATGCCCCTCTGC
HN187	TCAATTGCAATTTGTAAGAGAAGGGGAGAAAGACAAAATAATAATATGGGTAAAAAGCCT

Primer	Sequence (5' to 3')
HN188	GTTTTTTTCTTTAAAAAACTATGTATAATATAAAACATGCAATTTATTCCTTTGCCC T
HN189	CAAAAGTCTAGCAGCGAAAAGTACGCGAAGAATCTACTATAGATAATGGGTAAAAA GCCT
HN190	TCTTTTACAAAAAATCATTTTTGAAGGAAAATATAACGTTAATTTATTCCTTTGCCC T
HN191	AACCCTGACAGTGACACCCCGTTACACACTCACAATTAAGTAGGGATGGGTAAAAA GCCT
HN192	GACCAAATTGATCGGGATTTGTGATGTGGGATGATGGGGTGACGTTTATTCCTTTG CCCT
GTO95	GACCAAGAGACTTCATGGGAC
GTO96	GAATTCAAACCTGACTGCGC
GTO103	GGTTGCTGCTTTGGTTATTGA
GTO104	TTTTGACCCATACCGACCAT
GTO311	5'P ATGAAAAGGTCTACTGTGGATCGATGAGAA
GTO312	5'P CTTCAATTTTTCCAGAGTCATTAACC
GTO313	ATATCAGCGTGCGGACCTGGT
GTO314	AATTTACCATTCACATGTTTATAGTATACATTATAGTTCTC
GTO275	ATTCCTCGAGAACTCTGCTGCGCGCTGAA
GTO276	GAATCTGCAGGCGAGAACGACAATGGGATTGAG
GTO277	GAATCTGCAGAAACACACAAACGAGCAGTGTC
GTO278	GAATGCGGCCGCAAGTCAGTGTTGAATAACTGGAGTAG
HW015	ACTCGGATCCATGCGTCTACTTCGAAGAAACAT
HW016	ACTGCCATGGCTGAATACAAAATTACGTAATAATTGATCGT
B29	CGCGGATCCATGCGTCTACTTCGAAGA
B30	CATGGCGGCCGCTCACAATTCGTCGTGAGCGTAATCTGGAACATCATATGGGTAAT TTTGGTTCTTTTCATCTAATTC
B36	ATGCGCGGCCGCGAGACGCGAATTTTTCGAAG
B37	GCATGGATCCTGTTTTATATTTGTTGTAAAAAGTAGA

2.4 Culture media composition

Yeast peptone dextrose (YPD) (complete) and selective media were prepared as previously described [138]. YPD was prepared with Bacto-yeast extract (BD Difco™, catalog #212750), Bacto peptone (BD Difco™, catalog #211677), D-Glucose monohydrate (Duchefa Biochemie, catalog #G0802-1000) and

Bacto-agar (BD Difco™, catalog #204010) in the composition indicated in Table 4. Selective media was prepared with Bacto-yeast nitrogen base first without amino acids (BD Difco™, catalog #291930), then amino acids are added for the appropriate auxotrophic selection (Table 4). Amino acids were purchased from Sigma Aldrich and prepared as described in Table 5 and 6. Inositol-free selective media was made with inositol-free Bacto-yeast nitrogen base (MP Biomedical, catalog #4027-412). Choline supplemented media was made by preparing 1 M choline (Sigma Aldrich, catalog #C7527) and adding it to selective media at a final 1 mM concentration.

Table 4: Culture media and plate composition

Medium	Component	Composition
YPD	1% Bacto-yeast extract 2% Bacto-peptone 2% D-Glucose monohydrate Distilled water	10 g 20 g 20 g 1000 ml
YPD plates	1% Bacto-yeast extract 2% Bacto-peptone 2% D-Glucose monohydrate 2% Bacto-agar Distilled water	10 g 20 g 20 g 20 g 1000 ml
Selective media	0.67% Bacto-yeast nitrogen base 2% D-Glucose monohydrate Distilled water 10x amino acid mix (see Table 5) 100x His/ 100x Leu/ 100x Trp/ 20x Ura (see Table 6)	6.7 g 20 g make up to 1000 ml 100 ml Volume added to a 1x dilution based on auxotrophic selection
Selective media plates	0.67% Bacto-yeast nitrogen base 2% D-Glucose monohydrate 2% Bacto-agar Distilled water 10x amino acid mix (see Table 5) 100x His/ 100x Leu/ 100x Trp/ 20x Ura (see Table 6)	6.7 g 20 g 20 g make up to 1000 ml 100 ml Volume added to a 1x dilution based on auxotrophic selection

Table 5: 10x Amino Acid Mix (Selection)

	Stock Conc.	Final Conc. Wanted	Amount to add for 1 L final volume
Adenine Sulphate	Powder	0.6 g/L	0.6 g
Arginine • HCl	Powder	0.2 g/L	0.2 g
Tyrosine	Powder	0.3 g/L	0.3 g
IsoLeucine	Powder	0.3 g/L	0.3 g
Lysine • HCl	Powder	0.3 g/L	0.3 g
Phenylalanine	Powder	0.5 g/L	0.5 g
Glutamic Acid	Powder	1.0 g/L	1.0 g
Aspartic Acid	Powder	1.0 g/L	1.0 g
Valine	Powder	1.5 g/L	1.5 g
Threonine	Powder	2.0 g/L	2.0 g

	Stock Conc.	Final Conc. Wanted	Amount to add for 1 L final volume
Serine	Powder	2.0 g/L	2.0 g
ddH ₂ O	-	-	Adjust to 800 ml

Table 6: Individual 100x Amino Acid

	Stock Conc.	Final Conc. Wanted	Amount to add for 250 mL final vol.
Uracil • HCl*	Powder	3 g/L	0.75 g
Tryptophan**	Powder	8 g/L	2.0 g
Histidine • HCl	Powder	6 g/L	1.5 g
Leucine	Powder	8 g/L	2.0 g
Methionine	Powder	2 g/L	0.5 g

2.5 Genetic screen

The library used was the yeast deletion library [139]. Using the Synthetic Genetic Array (SGA) methodology [140], the reporter strains YGT1228 and YGT1202 were mated to the MAT α yeast deletion library containing a single gene deleted with Kan^R. In short, following mating and sporulation on nitrogen starved medium plates for 7 days, the MAT α cells were ultimately passaged onto SD plates containing geneticin sulphate (200 μ g/ml), hygromycin B (200 μ g/ml) and the toxic amino acid derivatives canavanine (100 μ g/ml) and thialysine (100 μ g/ml) to select for strains carrying either Ire1/ Δ LD and Kan-marked gene deletions. The genetic screen was condensed with the 384 Solid Pin Multi-Blot Replicator (V&P Scientific, San Diego, CA) and performed in 384-colony format until analysis. Cells were subsequently pinned from 384 spots on agar to four 96-well plates using the 96 Solid Pin Multi-Blot Replicator (V&P Scientific, San Diego, CA) and inoculated in 200 μ l YPD medium per well and grown overnight at 30 °C. An automated high-throughput sampler (HTS) connected to the LSRFortessa X-20 (BD, Franklin Lakes, NJ USA) was used to measure the relative levels of GFP and mCherry. The program FACSDIVA v 8.0 (BD, Franklin Lakes, NJ) was used to acquire data in .fcs file format. Files were read with the program FlowJo X 10.0.7r2 (FlowJo, LLC). GFP and mCherry were excited at 488 and 561 nm, collected through a 505 and 595 nm long-pass filters and a 530/30 and 610/20 band pass filter, respectively. Reporter GFP fluorescence levels were normalised to the constitutive TEF promoter driven mCherry expression to correct for non-specific GFP expression. The median readout from 10,000 cells was obtained. The log₂ GFP/mCherry ratio was normalised to WT levels from each plate and used as final sample's reporter level using Eq. 1.

$$\text{UPR reporter levels} = \log_2 \left[\left(\frac{\text{GFP}_m}{\text{mCherry}_m} \right) \times \left(\frac{1}{\frac{\text{GFP}_{WT}}{\text{mCherry}_{WT}}} \right) \right] \quad (1)$$

To generate network regions enriched for similar GO (gene ontology) biological process terms, hits with a log₂ fold change ≥ 1.5 was used as input into the open source software platform CytoScape as previously described [141].

2.6 Indirect immunofluorescence

Indirect immunofluorescence was performed as previously described [142]. In brief, cells were grown to early log phase in selective media, fixed by 3.7% formaldehyde treatment and permeabilised. Monoclonal mouse anti-HA (1:500), anti-Flag mouse M2 monoclonal antibody (1:500) and rabbit anti-Kar2 (1:1000) were used as primary antibodies. Mouse anti-dylight 488 (1:500) and rabbit anti-dylight 550 (1:500) were used as secondary antibodies. Samples were imaged with a Zeiss LSM 710 confocal microscope with a 100x 1.4 NA oil plan-Apochromat objective (Carl Zeiss MicroImaging). Images were analysed using ImageJ 1.48v.

2.7 Live- cell confocal microscopy

For live-imaging, yeast cells were grown to an exponential phase at 30 °C in 3 ml of selective media. Samples were treated with 10 mM DTT for 1 hour or inositol depleted for 4 hours. Cells undergoing inositol depletion were washed 6 times before transferring to inositol free media. 500 µl of cells in selective media were placed on slides coated with 10 mg/ml Concanavalin A (Sigma-Aldrich, St. Louis, MO) mounted onto Attofluor™ cell chambers (Thermo Fisher, Waltham, MA) and imaged at room temperature. Samples were imaged with a Zeiss LSM 710 confocal microscope with a 100x 1.4 NA oil plan-Apochromat objective (Carl Zeiss MicroImaging). Ire1 cluster analysis was performed as previously described [67]. For Ire1 cluster analysis, 8-bit images were set to image threshold of 60/255 to result in a black and white image identifying fluorescent Ire1-Venus clusters. These clusters were analysed using the 'analyse particles' function in ImageJ 1.48v, counting Ire1 particles of set criteria (size: 4-infinity px; circularity: 0-1; including holes). The number of cells were determined manually and dead cells were left out of the analysis.

2.8 Spotting Growth Assay

Cells were grown overnight in 3 ml of selective media at 30 °C and diluted to 0.2 OD₆₀₀/ml from which three 10-fold serial dilutions were prepared and spotted on selective plates (0.25 µg/ml tunicamycin or 1 mM choline were added to the plates when indicated). Plates were incubated at 30 °C until the appearance of colonies.

2.9 Alkaline carbonate extraction

Alkaline carbonate extraction was performed as previously described [143]. In brief, cells were grown in early log phase and the equivalent of 50 OD₆₀₀ of cells were harvested. Cells were resuspended in 10 mM sodium phosphate buffer pH 7.0, 1 mM PMSF and protease inhibitor cocktail (PIC) (Roche) and lysed by bead beating. An equal volume of 0.2 M sodium carbonate (pH 11.5) was added to cell lysates and incubated 30 min at 4 °C and spun down at 100,000 x *g* for 30 min, 4 °C. The pellet (membrane fraction) was solubilised in 3% SDS, 100 mM Tris-HCl, pH 7.4, 3 mM DTT and incubated at 95 °C for 10 min. Proteins from total cell lysate and supernatant fractions (collected from centrifuged lysate) were precipitated with 10% trichloroacetic acid (TCA) and spun down 30 min at 18,400 x *g*, 4 °C. Proteins were resuspended in TCA resuspension buffer (1 mM Tris, pH 11, 3% SDS) and incubated 10 min at 95 °C. Solubilised proteins were separated by SDS-PAGE and transferred to nitrocellulose for immunoblot analysis. Protein loading buffer was added to each fraction and separated by SDS-PAGE followed by immunoblot analysis.

2.10 Immunoblot

Cells were grown to an early log phase overnight at 30 °C. Tunicamycin was added to a final concentration of 2.5 µg/ml and incubated at 30 °C for 1 hour, when indicated. Harvested cells were resuspended in 10% TCA was added to resuspended cells followed by the addition of 0.5 mm zirconium beads. Cells were disrupted by two 30 seconds cycles. The lysate was transferred to a new tube and combined with a 10% TCA bead wash. The precipitate was pelleted by centrifugation and vortexed in TCA resuspension buffer (100 mM Tris pH 11, 3% SDS, 1 mM PMSF). The samples were incubated 10 min at 95 °C spun down at 15 min at 18,400 x *g*, 4 °C. A portion of the extract was separated by

SDS-PAGE using a 15% gel and transferred to a nitrocellulose membrane. The blots were probed with primary antibodies followed by secondary goat anti-mouse IgG-IRDye 800 (LI-COR Biosciences) and goat anti-rabbit IgG-IRDye 680 (LI-COR Biosciences, Lincoln, NE) antibodies. Membranes were washed in TBS and visualised with the Odyssey CLx imaging system (LI-COR).

2.11 qPCR

Cells were grown to an early log phase overnight at 30 °C. Tunicamycin was added to a final concentration of 2.5 µg/ml and incubated at 30 °C for 1 hour, when indicated. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Venlo, Netherlands) following manufacture's protocol. DNase treatment in columns was carried out with RNase-free DNase (Qiagen, Venlo, Netherlands) following the manufacturer's protocol. cDNA was synthesised from 2 µg of total RNA using RevertAid reverse transcriptase (Thermo Fisher, Waltham, MA) following manufacturer's protocol. SYBR Green qPCR experiments were performed following the manufacturer's protocol using a QuantStudio 6 Flex Real-time PCR system (Applied Biosystems, Waltham, MA). cDNA (30 ng) and 50 nM of paired-primer mix were used for each reaction. Relative mRNA was determined with the comparative Ct method ($\Delta\Delta C_t$) normalised to housekeeping gene *ACT1*. Oligonucleotide primers used are listed in Table 3.

2.12 β -galactosidase assay

The β -galactosidase assay was performed as previously described [132]. Typically, cells were grown to an early log phase overnight at 30 °C. Tunicamycin was added to a final concentration of 2.5 µg/ml and incubated at 30 °C for 1 hour, when indicated. Four OD₆₀₀ of cells were collected and resuspended in 75 µl of Z buffer (125 mM sodium phosphate pH 7.0, 10 mM KCl, 1 mM MgSO₄, 50 mM β -mercaptoethanol). An aliquot of 25 µl was transferred into 975 µl of ddH₂O and the absorbance was measured at 600 nm. To the remaining resuspension, 50 µl chloroform and 20 µl 0.1% SDS was added and the resulting mixture was vortexed vigorously for 20 seconds. The reaction started with 700 µl of 2 mg/ml of O-nitrophenyl- β -D-galactoside (ONPG, Sigma) in Z buffer. The reaction was quenched with 500 µl of 1 M Na₂CO₃, and the total reaction time was recorded. Samples were spun for 1 min at

maximum speed. Absorbance of the resulting supernatant was measured at 420 nm and 550 nm. The β -galactosidase activity was calculated using Eq. 2.

$$\text{Miller units} = \left\{ \frac{\text{OD}_{420} - 1.75 \times \text{OD}_{550}}{\left[t \times \left(\frac{V_A}{V_R} \right) \times \text{OD}_{600} \right]} \right\} \times 1000 \quad (2)$$

V_A is the volume of cells of the assay, V_R is the volume of cells used to read absorbance at 600 nm. The values were then normalised to the activity of WT.

2.13 Chromatin Immunoprecipitation (ChIP) and Quantitative Real-Time PCR (qPCR)

ChIP was performed as previously described [144-146]. Typically, cells were grown to an early log phase. Tunicamycin was added to a final concentration of 2.5 $\mu\text{g/ml}$ and incubated at 30 °C for 1 hour, when indicated. Forty OD_{600} of cells were collected, resuspended in 35 ml of selective, and fixed 20 min with 3.7% formaldehyde at 25 °C. The reaction was quenched by adding 400 mM glycine. After an incubation of 5 min, cells were washed once with ice-cold TBS and resuspended in SDS lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS). Cell lysates from these samples were sonicated for 8 cycles 10 seconds of 30% amplitude (Precellys 24, Bertin Instruments), with 50 seconds incubation on ice between intervals. Samples were diluted with the ChIP buffer (16.7 mM Tris-HCl pH 8.0, 167 mM NaCl, 1.1% Triton X-100, 0.01% SDS) to obtain a final concentration of 0.1% SDS. Forty microliters of protein G/salmon sperm DNA agarose beads and anti-HA in 1:500 dilution (Covance) were added followed by overnight incubation at 4 °C. Beads were washed twice with low salt wash buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS) and high salt wash buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.1% SDS), once with LiCl wash buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.25 M LiCl, 1% IGEPAL-CA630 (Sigma-Aldrich), 1% deoxycholic acid), twice with TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Bound Hac1-HA was eluted by incubating the beads 20 min with 250 μl elution buffer (1% SDS, 0.1 M NaHCO_3) at 30 °C and repeated once. NaCl was added to the combined elution to a final concentration of 0.3 M and incubated overnight at 65 °C. Released DNA fragments were purified using the QiAprep Spin Miniprep Kit according to manufacturer's protocol. Specific primers were designed approximately 75 bases up and downstream of the predicted UPRE motif (Table 3). The input DNA was diluted 100 times and ChIP DNA was pre-amplified 8 times with a primer mix prior to qPCR. Quantitative PCR was performed

following the manufacturer's protocol using a CFX Connect Real-Time PCR Detection System (Bio-Rad). The Ct value obtained was used to calculate fold enrichment between experimental sample and normalised input using Eq. 3 and 4.

$$\Delta Ct_{input} = Ct_{input} - \log_2(100) \quad (3)$$

$$\text{fold enrichment} = 2^{(\Delta Ct_{input} - Ct_{IP})} \times 100 \quad (4)$$

Results are representative of three biological replicates. *P* values were calculated using one-tailed Student's *t* test.

2.14 DNA Microarray

Cultures were grown to an OD₆₀₀/ml of 0.25 at 30 °C in selective synthetic complete media. The UPR was induced in WT cells by 1-hour incubation of 2 mM DTT when indicated. Cells were harvested from cultures at cell density of less than 0.5 OD₆₀₀/ml. Total RNA was extracted by the hot acid phenol method as previously described [147]. Total RNA was subsequently cleaned up using the RNeasy Mini Kit (Qiagen). RNA quality control was carried out using the Agilent RNA Nano 6000 Chip (Agilent Technologies). RNA was prepared from independent triplicate samples. Probe preparation and microarray construction and analysis were performed as previously described [147, 148]. Probes were prepared using the Low Input Quick Amp Labelling System with 100 ng of Total RNA as starting material following manufacturer's instructions, which included One-Color Microarray-Based Gene Expression Analysis Protocol Version 6.5 (Agilent Technologies), and were hybridized on a Custom Microarray Agilent GE 8x60 K array. Arrays were scanned using a high-resolution DNA Microarray Scanner, model G2505C (Agilent Technologies). Data were analysed using GeneSpring GX software (Agilent Technologies). Differentially expressed genes were deemed significant with fold-change > 1.5 and ANOVA *P* values < 0.05. UpSetR was used to compare and visualise set intersections of significantly upregulated genes in a matrix-style layout [149]. GO terms analysis of gene lists acquired from the intersections was performed with DAVID [150]. Heat map in the figure was generated using R studio. The DNA microarray data discussed in this publication has been deposited in NCBI Gene Expression Omnibus (GEO) under series number GSE131146.

2.15 Statistics

The error bars denote standard error mean (SEM), derived from at least three biological replicates. The *P* values were calculated using two-tailed Student's *t* test unless otherwise indicated in the figure legends. Scatter plots were plotted using Graphpad Prism 8.

Chapter 3. Results

3.1 High throughput genetic screen uncovers biological processes activating Ire1 through its lipid sensing domain

We sought to determine gene deletions that activate the UPR in Ire1 and Ire1 Δ LD expressing cells to shed light on biological processes to reveal unknown pathways important for UPR^{LBS}. Previously, 18 known UPR target genes were deleted, of which only 9 activated the UPR in Δ III Ire1 due to lipid bilayer stress [64]. While Δ III Ire1 retains its ability to sense misfolded proteins, Ire1 Δ LD eliminates activation by misfolded proteins [64]. We therefore employed Ire1 Δ LD to better understand cellular perturbations that activate the UPR in a luminal domain independent manner in a global context by a high throughput genetic screen (Figure 8, 11). To this extent, we genomically integrated both the *IRE1* and *IRE1 Δ LD* constructs tagged to the dominant selective marker Hygromycin (Hyg) into strains harbouring a UPR element (*UPRE*)-GFP reporter cassette and a constitutive translation elongation factor 2 (*TEF2*) promoter driving mCherry expression as previously reported [130]. The *UPRE* is a motif present in the promoter of Hac1 responsive genes therefore the measure of GFP fluorescence can be used as an output of UPR activation (Figure 6). The *TEF2*pr-mCherry expression enables correction of nonspecific expression changes such as differential progression in phases of the cell cycle [63].

Figure 8. Schematic representation of Ire1 and Ire1 Δ LD used in the study. Ire1 and Ire1 Δ LD contains the signal sequence (ss, black) from amino acid position 1 to 18. This is continued by the luminal domain (orange) from position 19 to 526. The transmembrane domain (TM, light grey bar) is located from position 527 to 555 of the protein. The cytoplasmic region of Ire1 is from position 556 to 1115 of the protein and contains the kinase (674-982, green) and RNase domain (983-1115, purple). In the Ire1 Δ LD strains, the luminal domain is truncated from amino acids 31-526 of the luminal domain.

We first tested the validity of both query strains for the high content genetic screen by measuring UPR activation when exposed to the ER stress-inducer dithiothreitol (DTT), a reducing agent that promotes the accumulation of misfolded proteins within the ER lumen [151]. UPR activation was induced at the time of measurement in the *IRE1* query strain when subjected to DTT treatment, as indicated by relative GFP fluorescence (Figure 9A). This induction of the UPR was further visualised with GFP positive cells in the *IRE1* query strains, by confocal microscopy (Figure 10A). This increase in GFP was absent in the *IRE1* query strain not subjected to UPR^{PT} (Figure 9A, 10A). In the *IRE1ΔLD* strain, the levels of GFP fluorescence upon DTT treatment were the same as basal levels of both untreated *IRE1* and *IRE1ΔLD* cells, indicating its inability to sense UPR^{PT} (Figure 9A, 10A). GFP activation was expectedly absent during UPR^{PT} in the *IRE1ΔLD* strain due to the lack of the misfolded protein stress sensing luminal domain (Figure 9A, 10A). As visualised by confocal microscopy, mCherry protein expression was consistent across both query strains *IRE1* and *IRE1ΔLD* indicating proper functioning of background cellular processes such as cell cycle progression across the cells (Figure 10A).

Figure 9. The luminal domain of Ire1 is necessary for UPR^{PT} but not for UPR^{LBS} activation. (A) *IRE1* cells are GFP positive but not *IRE1ΔLD* cells during DTT treatment as measured by flow cytometry. Histogram representing the relative GFP fluorescence of *IRE1* and *IRE1ΔLD* non-treated cells (control, black) or treated with 10 mM DTT (green) for 1 hour. The numerical values represent the

mode of each sample. (B) The UPR is activated independently of the luminal domain in the *get1Δ*, *spc2Δ*, *scj1Δ*, and *ste24Δ* mutants. Query strains *IRE1* (blue) and *IRE1ΔLD* (red) with genomically integrated *UPREpr-GFP* and *TEF2pr-mCherry* carrying single deletions of either 4 genes: *SCJ1*, *SPC2*, *GET1* or *STE24*. Cells were grown to mid-log phase before GFP and mCherry fluorescence was measure by flow cytometry. The numerical values represent the mode of each sample.

Figure 10. The luminal domain is required for UPR^{PT} activation but not UPR^{LBS} activation in *scj1Δ* and *spc2Δ* mutants. (A) Query strains *IRE1* and *IRE1ΔLD* with genomically integrated *UPREpr-GFP* and *TEF2pr-mCherry* were incubated 1h with 10 mM DTT, when indicated, before visualisation by confocal microscopy. (B) UPR^{LBS} is activated upon the deletion of *SCJ1* and *SPC2*. Query strains *IRE1* and *IRE1ΔLD* with genomically integrated *UPREpr-GFP* and *TEF2pr-mCherry* with either *SCJ1* or *SPC2* deletions were grown to mid-log phase before visualisation by confocal microscopy. Scale bar: 5 μm. At least 30 cells per condition of randomly chosen fields were imaged for analysis for each replicate. The represented images are of 3-4 cells per condition.

Next, to ensure that the UPR is functional in the *IREΔLD* query strain, since GFP fluorescence was undetectable by DTT treatment in these cells, we deleted *STE24*, *SCJ1*, *GET1* and *SPC2*, previously screened for UPR^{LBS} activation in Δ III *Ire1* [64]. *Scj1* functions as Hsp40/DnaJ co-chaperone of the essential ER chaperone *Kar2* and *Spc2* as part of the signal peptidase complex necessary for maintaining ER homeostasis [152, 153]. *Ste24* was recently found to be required to relieve clogging at

the translocon by the cleavage of obstructive proteins thereby alleviating ER stress [154]. Contrary to the published findings, where UPR activation was compromised in Δ III Ire1 [64], we observed UPR activation in *ste24* Δ , *scj1* Δ and *spc2* Δ cells expressing Ire1 Δ LD (Figure 9B). Although *SCJ1*, *SPC2* or *STE24* deletions activate the UPR likely due to protein aberrancies [50, 132, 154], our data suggest that the presence of cross-talk between protein folding, targeting, proteolysis and lipid homeostasis is sensed by the UPR in *S. cerevisiae* (Figure 9B, 10B). Indeed, the link between *N*-glycosylation and lipid metabolism was very recently revealed, when the mutation of *SEC59*, a dolichol kinase required for *N*-glycosylation resulted in the upregulation of genes involved in phospholipid, TAG, fatty acid and sterol biosynthesis [155]. Lipid composition could modulate both protein folding and/or protein trafficking through the ER, further establishing the role of these genes in UPR^{LBS} upon their deletion. In addition, it is important to note that Spc2, Ste24 and Get1 are all ER integral membrane proteins and the loss of these proteins leads to the accumulation of aberrant proteins consequently perturbing the ER bilayer leading to the activation of the *IRE1* and *IRE1* Δ LD cells. Since *GET1* is required for the proper insertion of tailed-anchored proteins into the ER membrane, the loss of *GET1* impairs tail-anchored protein biogenesis and results in the improper insertion of tail-anchored proteins into the ER [156]. This may not only result in the activation of the UPR but also destabilise the ER membrane explaining UPR activation observed in Δ III, *IRE1* and *IRE1* Δ LD cells.

In our analysis, *IRE1* and *IRE1* Δ LD *scj1* Δ cells had higher GFP fluorescence compared to *spc2* Δ , *get1* Δ and *ste24* Δ cells (Figure 9B). This can be possibly attributed to Scj1's function as a Hsp40 co-chaperone to the Hsp70 chaperone Kar2 in the ER and is therefore important for the Kar2 functional cycle [152] (described in Section 1.1). As previously described, co-chaperones of BiP play an indirect role in Ire1 α activation. Given that Kar2 is homologous to BiP, it is likely Scj1 plays a yet defined role in the Kar2 mediated regulation of Ire1, the deletion of which causes potent UPR activation. *SCJ1* is a known UPR target gene and our transcriptomic data shows that *SCJ1* is 1.5-fold upregulated in WT cells in the presence of DTT (data deposited in NCBI GEO database GSE131146). Since Kar2 fine tunes the UPR, the loss of *SCJ1* may negatively affect Kar2 protein folding capacity important for ER homeostasis therefore leading to higher ER stress in *IRE1* and *IRE1* Δ LD cells [152] (Figure 9B). Furthermore, ERAD is dependent on the Hsp70 and Hsp40 subset of chaperones [157] therefore it may be likely that *scj1* Δ impairs the ERAD in the absence of UPR^{PT} [132] and contributes to the UPR^{LBS} due to changes in cellular lipidome thereby activating *IRE1* Δ LD cells [158] (Figure 9B). Indeed, sterol

homeostasis has been shown to be regulated by ERAD [159]. Although *GET1*, *SPC2* and *STE24* are all part of the cellular proteostasis network associated to the ER membrane, the loss of *SCJ1* suggests that perturbation to chaperones seems to indirectly activate UPR^{LBS} more drastically, through membrane associated defects. This hints at the intimacy between UPR^{PT} and UPR^{LBS} and the complexity of the regulation of UPR genes in an Ire1 luminal domain-dependent or independent manner. In conclusion, our data suggests luminal domain independent activation of the UPR, further validating the use of the *IRE1* Δ LD query strain to uncouple UPR^{PT} and UPR^{LBS} (Figure 9B, 10B).

We next performed the genetic screen by mating the *IRE1* and *IRE1* Δ LD query strains to the genome wide deletion library, a method adapted from the Schuldiner Lab [63]. Mated diploids undergo a series of sporulation and selection steps to end up with haploid meiotic progenies of *IRE1* or *IRE1* Δ LD containing single gene deletions (Figure 11, see section 2.5 for method). After the final selection step, cells were grown overnight in YPD rich media to allow for growth of the cells to mid-log phase. The UPR output was measured as GFP and mCherry fluorescence of 10,000 cells per sample using a 96-well format high-throughput flow cytometry. UPR activation was quantified as a normalised ratio of the median of GFP/mCherry fluorescence. Additional genes were excluded if cell count per sample was less than 350, suggesting growth defect [63].

Figure 11. Genetic screen methodology to identify gene deletions that activate UPR^{LBS} . The *IRE1* and *IRE1* Δ LD query strains were crossed to the non-essential gene deletion library. Mated diploid heterozygotes undergo sporulation and a series of selection to isolate haploid single mutant progenies of *IRE1* or *IRE1* Δ LD *UPRE*-GFP and *TEF2pr*-mCherry cells. UPR induction was measured through a flow cytometer in a high throughput 96 well format and median single cell fluorescence of GFP and mCherry levels were read. UPR activation was quantified as the median single cell fluorescence

(GFP/mCherry) levels per sample was normalised to the median (GFP/mCherry) levels per plate. This method of high content genetic screening was adapted from Jonikas *et al.* [63].

Out of 4,847 genes screened, we identified 629 (blue) and 958 (green) genes whose deletion significantly upregulated the UPR ($\log_2$ fold change ≥ 1.5) in *IRE1* or *IRE1 Δ LD* cells respectively (Figure 12, refer to Appendix Table S1, S2 for a list of all hits). Compared to *IRE1* cells, UPR activation observed in *IRE1 Δ LD* cells is luminal domain independent. The 448 non-overlapping gene deletions that activated the UPR in *IRE1* cells (blue) may indicate their importance in maintaining proteostasis within the cell. Previously, using the SGA methodology, Jonikas *et al.* identified 399 gene deletions that upregulated the UPR [63]. As validation of our data, we compared the 448 gene deletions that activated the UPR in *IRE1* cells against these 399 known gene deletions [63]. There was a sizeable overlap of genes including genes representing proteins that are highly enriched throughout the secretory pathway, including the ER, Golgi, vacuole and endosomes (Table S1). Gene deletions linked to *N*- and *O*-glycosylation, ERAD, lipid biosynthesis and multiple steps of vesicular trafficking similarly led to UPR induction [63] (Table S1). Due to the shared transmembrane (TM) domain of *IRE1* and *IRE1 Δ LD* expressing cells, we expected gene deletions of *IRE1 Δ LD* cells to be a subset of those identified in *IRE1* cells. Surprisingly, there was a lack of overlap (only 181 genes) between these two gene deletion subsets. We attributed this to the disruption of cellular processes that results from the removal of the stress sensing domain (LD) of Ire1. Previously, associators of the Ire1 luminal domain, functioning as Ire1 regulators were reported [160-163] (this is further discussed in Section 4.2). The 777 gene deletions (green) that activate the UPR in *IRE1 Δ LD* cells possibly contain uncharacterised protein interactors of the luminal domain buffering Ire1 activity. These gene deletions may activate the UPR due to the loss of inhibitory mechanisms of Ire1 or conversely, cause the hyperactivation of Ire1. We also propose that another possible explanation for the higher number of hits obtained in *IRE1 Δ LD* cells is due to higher basal UPR activation in *IRE1 Δ LD* compared to *IRE1* cells. The resulting minor overlap of 181 genes may reflect the intimacy between Ire1 luminal domain and ER homeostasis (Figure 12, red). These 181 gene deletions that activated the UPR in *IRE1* and *IRE1 Δ LD* cells suggests that they are lipid bilayer stress related (Figure 12, refer to Appendix Table S3 for a list of all hits). Since both *IRE1* and *IRE1 Δ LD* sense lipid bilayer stress, we looked further into this subset of genes to better understand biological processes buffering changes during UPR^{LBS}.

Figure 12. A subset of 181 genes are required for ER membrane homeostasis. Venn diagram shows 448 gene deletions activate the UPR in *IRE1* cells (blue), 777 activated the UPR independently of the luminal domain in *IRE1ΔLD* cells (green) and an overlap of 181 gene deletions activated the UPR in both *IRE1* and *IRE1ΔLD* expressing cells (red).

GO term enrichment analysis from the 181 genes reveal that processes such as protein glycosylation and mannosylation, multiple steps of vesicular trafficking, ERAD and lipid metabolism are required for ER homeostasis in both *IRE1* and *IRE1ΔLD* cells (Figure 13, 14). Perturbation of ER homeostasis by the deletion of these genes cause either a direct or membrane-associated indirect effect on UPR activation that is required to buffer changes to the membrane bilayer. Amongst the 181 genes, we found *SCJ1*, *STE24*, *GET1* and *OPI3*, previously tested to activate UPR^{LBS} in *IRE1ΔLD* cells [64]. This indicates the deletion of these genes activate the UPR through ER membrane or lipid associated abnormalities since they similarly activate the UPR in the both the ΔIII and the *IRE1ΔLD* strain [63] (Figure 9B, 10B).

Figure 13. Biological processes related to ER membrane homeostasis. The genes enriched for functional clusters of *IRE1* only (blue), *IRE1ΔLD* only (green) and the overlap *IRE1* and *IRE1ΔLD* (red). Only gene deletions that upregulate the UPR ≥ 1.5 -fold are represented (see Tables S1, S2 and S3 in Appendix for a complete list of genes).

Figure 14. Genetic interactions belonging to protein folding, vesicle traffic, peroxisome and metabolism and fatty acid synthesis. The dots represent genes perturbing ER homeostasis in *IRE1* only (blue), *IRE1ΔLD* only (green) and the overlap of *IRE1* and *IRE1ΔLD* (red) upon their deletion. The

networks identified within these clusters give an indication that these processes may be implicated in UPR^{LBS} in *IRE1* and *IRE1ΔLD* cells.

Figure 15. Deletion of genes coding proteins intimately linked to the ER membrane activate the UPR. Heatmap representing the degree of UPR activation caused by the deletion of selected genes in *IRE1* and *IRE1ΔLD* cells. These genes have functions associated to the proteostasis network, multiple steps of vesicular trafficking fatty acid and PC biosynthesis. ER stress occurs possibly through membrane associated aberrancies causing UPR activation. These genes were selected based on their gene ontology functions (ER membrane associated), involvement in the UPR and on the degree of UPR activation. Lipid biosynthetic genes *PCT1*, *OPI3* and *CHO2* required for PC biosynthesis activated the UPR upon their deletion. Heatmap represents the log₂ fold change of UPR reporter levels upon gene deletion. Table 7 describes the full functions of the genes listed in this figure (see below). Refer to Table S3 of Appendix for the complete set of hits.

Table 7: Function of genes represented in the heatmap above (Figure 15)

Gene	Function
Protein folding, insertion into the ER and proteolysis	
<i>SCJ1</i>	peptidase required for the signal peptide cleavage and proper localisation of proteins into the ER [164].
<i>SPC2</i>	a DnaJ co-chaperone of the Hsp70 chaperone Kar2 [153].
<i>STE24</i>	zinc metalloprotease required for the cleavage of clogged proteins at the translocon [165].
<i>GET1</i>	part of the Golgi apparatus to ER traffic (GET) complex required for vesicle-mediated Golgi to ER transport and protein insertion to the ER membrane [156].
Protein mannosylation and glycosylation	
<i>PMT2</i>	Protein O-mannosyltransferase of the ER membrane, important for endoplasmic reticulum quality control (ERQC) [166, 167].
<i>PMT3</i>	Paralog of <i>PMT2</i> [168].
<i>PMT6</i>	Protein O-mannosyltransferase of secretory proteins [169].
<i>ALG9</i>	Involved in N-linked glycosylation [170].

Table 7: Function of genes represented in the heatmap above (Figure 15)

Gene	Function
Vesicular trafficking	
<i>VPS8</i>	GTPase binding protein of the class C core vacuole/endosome tethering (CORVET) endosome tethering complex for endosome to vacuole protein targeting [171].
<i>VPS29</i>	Endosomal retromer complex, involved in retrograde transport of cargo proteins from endosomes to the Golgi apparatus [172]
<i>VPS63</i>	Unknown function, involved in vacuolar protein sorting [173].
<i>VPS72</i>	Required for vacuolar protein sorting [173]
<i>TLG2</i>	Part of the t-SNARE (SNAP-Receptor) complex, required to mediate fusion of endosome-derived vesicles with the Golgi [174].
<i>SEC22</i>	Part of the SNARE complex shuttling between the ER and Golgi [175, 176].
ERAD	
<i>HRD1</i>	Ubiquitin-protein ligase involved in ERAD [177].
<i>HRD3</i>	Forms the HRD complex with Hrd1 for ERAD [177].
Fatty acid synthesis	
<i>OAR1</i>	May comprise of a type-III mitochondrial fatty acid synthase with Mct1, involved in fatty acid metabolism [178]
<i>MCT1</i>	Putative component of a type-II mitochondrial fatty acid synthase involved in fatty acid metabolism [178].
Phosphatidylcholine synthesis	
<i>PCT1</i>	an enzyme of the CDP-choline pathway for phosphatidylcholine synthesis [179].
<i>OPI3</i>	Phospholipid methyltransferase of the CDP-DAG pathway catalysing the final two steps of PC biosynthesis [180].
<i>CHO2</i>	Phospholipid methyltransferase of the CDP-DAG pathway catalysing the first step of PC biosynthesis [180].

Genes that are closely related to the ER were identified within these the overlapping subset of 181 genes (Figure 14, 15). Selected genes that are ER associated and have been described to be involved in UPR activation have been curated in Figure 15 and the gene ontology function described in Table 7. For example, the deletion of genes required for protein glycosylation and mannosylation highly activated the UPR in both *IRE1* and *IRE1ΔLD*. Alg9, an enzyme required for the addition of mannose residues for *N*-linked glycosylation and the protein-mannosylation proteins Pmt2, Pmt3 and Pmt6 are required for ER homeostasis in both *IRE1* and *IRE1ΔLD* cells [181, 182] (Figure 15, Table 7). *PMT* family members are implicated in ER homeostasis since the inhibition of *O*-mannosylation results in the accumulation of non-glycosylated misfolded proteins, perturbing ER homeostasis and upregulating

UPR target genes [182, 183]. *PMT1* deficiency was also shown to cause UPR activation [184]. In addition, genome wide analysis during UPR activation in *S. cerevisiae* indicate that the expression of *PMT1*, *PMT2*, *PMT3* and *PMT5* are influenced by the UPR [50, 185]. Other cellular processes related to the membrane include vesicular trafficking such as the *TLG2* gene, required for endo-lysosomal fusion and the *VPS* (*VPS8*, *VPS29*, *VPS61*, *VPS63*, *VPS72*) family of genes for endosomal to Golgi transport are required for ER membrane integrity in *IRE1* and *IRE1ΔLD* cells [171, 186] (Figure 15, Table 7). Another important cellular process associated to UPR^{LBS} maintenance is the ERAD machinery. Hrd1, an E3 ubiquitin ligase and Hrd3, a Hrd1 associated protein, together required for Hrd-dependent ER degradation, was found to be necessary for ER homeostasis in *IRE1* and *IRE1ΔLD* cells (Figure 15, Table 7). Hrd1 was suggested to have additional function of forming a ubiquitin-gated protein conducting channel for the retro-translocation of misfolded luminal protein domains across a lipid bilayer to cope with misfolded proteins [187]. Given that Hrd1/Hrd3 contacts the ER membrane bilayer, there could be a potential of this complex in effecting changes to the lipid bilayer to buffer UPR^{LBS}. This is in addition to the protective role of Hrd1 during UPR^{PT} by degrading misfolded protein accumulated in the ER.

The deletion of lipid metabolic genes caused UPR activation in both *IRE1* and *IRE1ΔLD* cells (Figure 15, Table 7). *MCT1* and *OAR1*, a malonyl-CoA:ACP transferase and 3-oxo-acyl-((acyl carrier protein) reductase) were identified to be putative components of the type-II mitochondrial fatty acid synthase, involved in mitochondrial phospholipid remodelling [178] (Table 7). The upregulation of the UPR upon the deletion of *MCT1* and *OAR1* suggests mitochondrial membrane remodelling occurring as part of the global homeostatic adaptation. Most significantly, genes previously implicated in membrane stress response, *CHO2* and *OPI3* activated the UPR upon their deletion, as measured by an increase in its downstream target, Kar2 (Figure 15, Table 7) [188]. The lack of *OPI3* highly upregulated the UPR in both *IRE1* and *IRE1ΔLD* query strains (Figure 15, 16), and the deletion of its homolog *Pemt* has been shown to activate the UPR due to the disruption of ER membrane homeostasis of the ER [121]. *OPI3* is required for *de novo* synthesis of abundant biological membrane building block, PC, and its deletion causes a dramatic shift in PE/PC ratio [65]. This increase in PE/PC ratio causes membrane lipid disequilibria that is dependent on the UPR for its survival [65]. Similarly, a decrease in PE/PC ratio has been observed in NAFLD mice model, causing a loss in hepatocytes membrane integrity leading to the disruption of the SERCA pump which consequently activates the UPR [121]. The decrease in PE/PC

ratio was probably due to the upregulation of choline-phosphate cytidyltransferase (*Pcty1a*) and phosphatidylethanolamine *N*-methyltransferase (*Pemt*, homologue of *OPI3*) in a fatty liver (Figure 3). Patients with the more severe form of NALFD, non-alcoholic steatohepatitis (NASH) also exhibit similar decrease in PE/PC ratio [118]. Regardless, a deviation from the optimal PE/PC ratio within ER membrane leads to the activation of the UPR. We sought to use *opi3Δ* as a model of lipid bilayer stress (LBS) in our *IRE1* and *IRE1ΔLD* cells to uncouple UPR^{PT} and UPR^{LBS} for a better understanding of the mechanism of UPR^{LBS} sensing.

Figure 16. PC depletion activates the UPR independently of the luminal domain. Query strains *IRE1* and *IRE1ΔLD* with genomically integrated *UPREpr-GFP* and *TEF2pr-mCherry* deleted of *OPI3* were grown to mid-log phase before visualisation by confocal microscopy. Scale bar: 5 μm. At least 30 cells per condition of randomly chosen fields were imaged for analysis for each replicate. The represented images are of 3-4 cells per condition.

3.2 Ire1 and Ire1ΔLD are properly integrated into the ER membrane during PC depletion

The removal of the luminal domain of Ire1 may alter its localisation and integration within the ER membrane within cells. It was therefore important to ensure the proper localisation and integration of Ire1 and Ire1ΔLD (mentioned in the figures and text interchangeably as IΔLD for simplicity) into the ER membrane to exclude the possibility of non-specific activation of ER stress due to the mis-localization of Ire1ΔLD in all our experiments. We first assessed its subcellular localisation by indirect

immunofluorescence. Like wildtype HA-tagged Ire1, Ire1 Δ LD-HA is distributed to both nuclear and peripheral ER as observed by the staining pattern in *ire1* Δ and *ire1* Δ *opi3* Δ mutants colocalizing with the ER Hsp70 chaperone Kar2 (Figure 17).

Figure 17. The luminal domain is not required for the localisation of Ire1 into the ER membrane. Indirect immunofluorescence of *ire1* Δ and *ire1* Δ *opi3* Δ mutants expressing Ire1-HA and I Δ LD-HA (Ire1 Δ LD-HA) show co-localisation with endogenous Kar2 as an ER marker. Scale bar: 5 μ m. At least 30 cells per condition of randomly chosen fields were imaged for analysis for each replicate. The represented images are of 3 cells per condition.

We further validated the proper integration of Ire1 Δ LD into the ER membrane by an alkaline carbonate extraction derived from the membrane fraction of cells after whole cell lysis. The alkaline carbonate extraction method disrupts vesicles to form open sheets and results in the release of the contents of the membrane fraction as well as peripheral proteins into the soluble fraction [143]. Integral proteins remain embedded in the membrane and remains insoluble. Both Ire1 and Ire1 Δ LD in *ire1* Δ and *ire1* Δ *opi3* Δ mutants were identified in the pellet fraction indicating proper membrane integration (Figure 18). ER translocon subunit Sec61 and Kar2 were used to detect transmembrane and membrane soluble proteins, respectively (Figure 18). Kar2 associates to ER membrane proteins for functions such as ERAD and protein translocation [32, 189, 190]. The relative abundance of this ER luminal protein and its association to membrane proteins results in residual detection in the insoluble fraction (Figure 18).

Figure 18. Ire1 and Ire1 Δ LD are properly integrated into the ER membrane. Ire1 and Ire1 Δ LD in *ire1 Δ* and *ire1 Δ opi3 Δ* cells were detected in the pellet (P) fraction after alkaline carbonate extraction as detected by western blot. This indicates that Ire1 and Ire1 Δ LD are integrated into the membrane. Soluble cytosolic protein Kar2 is released into the supernatant. T, total lysate; S, supernatant; P, pellet. The asterisk (*) indicates a non-specific band.

3.3 PC depletion activates the UPR independently of Ire1 luminal domain

The luminal domain of Ire1 plays an essential role in sensing ER stress caused by the accumulation of misfolded proteins in the ER lumen. In *S. cerevisiae*, compromising mutations and the substitution of the misfolded protein binding groove of Ire1 by a non-ligand binding bZIP dimer groove activated the UPR upon membrane lipid bilayer stress from inositol [64]. Similarly, in mammalian cells, Ire1 lacking the luminal domain was sufficient to detect lipid bilayer stress at the membrane resulting in UPR^{LBS} [66]. A spotting assay was first performed to determine the effect of Ire1 and Ire1 Δ LD on the growth of cells during UPR^{PT} and UPR^{LBS}. The *N*-linked glycosylation inhibitor tunicamycin was used to induce the UPR by the accumulation of misfolded proteins in the ER. Both *IRE1 ire1 Δ* and *IRE1 ire1 Δ opi3 Δ* mutants grew under ER stress when treated with 0.125 μ g/ml tunicamycin (Figure 19). In general, viability of all the strains decreased due to the strenuous effect of tunicamycin (Figure 19). Removal of the luminal domain in *ire1 Δ* mutants eliminates the ability of Ire1 to sense misfolded proteins decreasing cell viability as visualised by slower growth compared to non-stressed conditions (Figure 19). This observation agrees with the lack of GFP expression in *IRE1 Δ LD* cells (Figure 9A, 10A). In comparison, *I Δ LD ire1 Δ opi3 Δ* cells remained viable, although the cells appear to be more sensitive to protein stress (Figure 19). The effect of slow growth due to PC depletion is rescued when *IRE1* and *I Δ LD ire1 Δ opi3 Δ* cells were spotted onto choline supplemented plates (Figure 19). This suggests that alternatively, lipid

bilayer stress activates Ire1, independent of protein stress, for the upregulation of downstream homeostatic genes that enables cells to adapt to UPR^{LBS} and survive. Additionally, this lipid-induced activation of the UPR was enough to rescue the cells from misfolded protein stress.

Figure 19. Ire1 lacking its luminal domain is sufficient to rescue cells from UPR^{LBS}. *ire1Δ* and *ire1Δopi3Δ* mutants carrying *IRE1* and *ΔLD* (*IRE1ΔLD*) were grown in synthetic complete selective medium, diluted to 0.2 OD cells, 10 times serially diluted and spotted onto untreated (carrier), 0.125 μg/ml tunicamycin (Tm) plates or 1 mM choline plates. Plates were incubated at 30°C for 2 days.

Upon ER stress, *HAC1* pre-mRNA (*HAC1* unspliced or *HAC1^u*) is spliced by Ire1 to produce a mature mRNA *HAC1* (*HAC1* spliced or *HAC1^s*) that is subsequently translated into a basic leucine zipper (bZIP) transcription factor. The activity of Ire1 was measured by the splicing of mRNA *HAC1*. *HAC1^s* was detected during both UPR^{PT} in DTT treated *IRE1 ire1Δ* cells as well as during UPR^{LBS} in *IRE1* and *ΔLD ire1Δopi3Δ* mutants (Figure 20A, 20B). Interestingly, *HAC1^s* is significantly higher ($p=0.190$) in *IRE1 ire1Δopi3Δ* mutants (47%) compared to *IRE1 ire1Δ* cells (40.1%), suggesting a stronger UPR during LBS (Figure 20B). *HAC1^s* was undetectable in *ΔLD ire1Δ* cells indicating that the Ire1 luminal domain is essential to detect protein-induced ER stress (Figure 20A, 20B). The effect of UPR^{LBS} on *HAC1* mRNA splicing can be rescued by choline supplementation, indicating the specificity of PC depletion on the activation of the UPR (Figure 20A, 20B).

Figure 20. The luminal domain is not required for *HAC1* mRNA splicing upon PC depletion. (A) *IRE1* and *ΔLD* (*IRE1ΔLD*) *ire1Δ* mutants were left uninduced (no DTT) or induced (2 mM DTT) and *IRE1* and *ΔLD* (*IRE1ΔLD*) *ire1Δopi3Δ* mutants were left untreated or treated with 1 mM choline (cho) overnight. *HAC1* mRNA was detected by RT-PCR. The two forms of *HAC1* are indicated as *HAC1^U* (unspliced) and *HAC1^S* (spliced). (B) Quantification of the *HAC1^S* over total *HAC1* in (A) by ImageJ 1.48v confirms *HAC1* splicing in *ire1Δopi3Δ* mutants independent of Ire1 luminal domain. Error bars represent the standard error mean of three biological replicates.

Upon UPR initiated translation, Hac1 binds to UPR elements (*UPRE*) within the promoter of a subset of UPR-upregulated genes. One such motif is the *UPRE-1* motif, a nucleotide sequence of 22 base pairs present in the promoter of several UPR-related genes such as the ER resident chaperone Kar2 and PDI [191]. To determine the relative activity of the UPR during UPR^{LBS} , a β -galactosidase reporter assay was created by adding *UPRE-1* to the *CYC1* promoter followed by the *LACZ* gene [4, 5]. To monitor β -galactosidase activity by spectrophotometry, the ortho-Nitrophenyl- β -galactoside (ONPG), an analogue of lactose, was used.

The UPR was activated upon tunicamycin treatment in *IRE1 ire1Δ* cells. Expectedly, this activation was absent in tunicamycin treated *ΔLD* (*IRE1ΔLD*) *ire1Δ* cells (Figure 21). Interestingly, a stronger UPR induction ($p=0.0238$) was observed during UPR^{LBS} in *IRE1* (26.8%) compared to *ΔLD* (12.9%) *ire1Δopi3Δ* cells (Figure 21). This corresponds to the similar difference observed during *HAC1* splicing in the same strains *IRE1 ire1Δopi3Δ* cells (47%) versus *ΔLD ire1Δopi3Δ* cells (32.1%) ($p=0.0075$,

Figure 20B). This suggests that the higher UPR activation and *HAC1* splicing observed in *IRE1 ire1Δopi3Δ* cells may be due to cumulative activation of the UPR from misfolded proteins accompanying lipid bilayer stress. Without the Ire1 luminal domain, UPR activity is significantly decreased, suggesting that in addition to its misfolded proteins stress sensing function, the luminal domain may have a secondary role in the efficient sensing of UPR^{LBS} (Figure 20B, 21). Similarly, in full length Ire1α cells subject to LBS, the UPR response is stronger compared to ΔLD-Ire1α cells indicating that the contribution of UPR^{LBS} to UPR^{PT} is sensed through the luminal domain and the effect of LBS on the UPR is additive in Ire1 expressing cells [66]. This effect of lipid bilayer stress is rescued by the supplementation of choline for PC synthesis via the CDP-DAG pathway (Figure 3). This restores PE/PC balance within the cells which suggests that ER membrane homeostasis within the cell is required for protein homeostasis within the ER lumen.

Figure 21. The luminal domain is not required for UPR^{LBS}. *ire1Δ* mutants carrying *IRE1* and Δ LD were left uninduced (no tunicamycin) or induced with 2.5 μg/ml tunicamycin (Tm). *ire1Δopi3Δ* mutants carrying *IRE1* and Δ LD were left untreated (no choline) or treated with 1 mM choline (cho). The strains were assayed for β-galactosidase activity and normalised to untreated *ire1Δ* carrying *IRE1*. Error bars represent the standard error mean of three biological replicates.

To determine other cellular processes that are compensating for UPR^{LBS}, we looked at the cytosolic stress response pathway called the heat shock response (HSR), responsible for maintaining cytosolic proteostasis [192]. Targets of the HSR are activated during lipid disequilibrium raising the concern of

the role of the HSR during lipid bilayer stress. When the HSR is activated, heat shock proteins are upregulated to restore proteostasis and relieve stress to the cell [193]. To measure the activation of the HSR to exclude the possibility that the effect of LBS on the UPR is influenced by the HSR, a similar method of fusing a heat shock element (*HSE*) to the *LACZ* reporter gene was used.

The effect of heat shock (HS) on the cells could be seen by the activation of the HSR when *IRE1 ire1Δ* mutants were subjected to 37°C for 1 hour (Figure 22). The HSR was activated in *ΔLD ire1Δ* mutants similar to wild-type HS levels, suggesting a possible compensatory response when cells are expressing *ΔLD* (Figure 22). In PC depleted *ire1Δopi3Δ* cells however, the HSR was strongly induced in both *IRE1* and *ΔLD* cells in the absence of heat shock, possibly due to drastic changes to the ER membrane composition due to PC depletion. The ER stress response has been previously associated to the cytosolic stress response [194]. Since the strength of the HSR response is similar in *ΔLD* and *IRE1 ire1Δopi3Δ* cells, this suggests that the HSR did not play a role in alleviating the decrease in UPR activation observed in *ΔLD ire1Δopi3Δ* cells (Figure 22).

Figure 22. The luminal domain is necessary to maintain cytosolic homeostasis. *IRE1 ire1Δ* mutants were left untreated or heat shocked (HS) at 37°C for 1 hour. *ΔLD ire1Δ* mutants, *IRE1* and *ΔLD ire1Δopi3Δ* mutants were left untreated. The strains were assayed for β- galactosidase activity and normalised to untreated *IRE1 ire1Δ* cells. Error bars represent the standard error mean of three biological replicates.

3.4 The overexpression of the luminal domain uncouples UPR^{PT} from UPR^{LBS}

To further support the data shown above that the luminal domain is not required for Ire1 activation during UPR^{LBS}, we performed an assay to titrate out misfolded proteins by overexpression of the luminal domain. The luminal domain was fused to the promoter of the highly expressed gene *PGK1* with an N-terminal Ire1 signal peptide for ER localisation and conjugated to the ER retention signal HDEL at the C-terminus. We first confirmed the proper localisation of the LD into the ER by indirect immunofluorescence (Figure 23A). Additionally, we probed for Ire1^{LD}-HA transformed into wild-type cells to determine the expression levels of Ire1^{LD} (Figure 23B). Ire1^{LD}-HA was detected at high levels in WT lysate at the correct molecular weight of 60 kDa (Figure 23B, lane 2). The treatment of cells to tunicamycin resulted in an unknown lower band potentially representing the non-glycosylated band of the luminal domain, as previously shown in Liu *et al.* [195] (Figure 23B, lane 3).

Figure 23. Ire1 luminal domain (Ire1^{LD}) is properly localised to the ER. (A) Indirect immunofluorescence of WT cells expressing HA tagged luminal domain (LD) with endogenous Kar2 as an ER marker. Scale bar: 5 μ m. At least 30 cells per condition of randomly chosen fields were imaged for analysis for each replicate. The represented images are of 2 cells per condition. (B) Overexpressed Ire1 luminal domain (Ire1^{LD}) is detected by western blot. A 60 kDa band corresponding to monomeric Ire1 LD was detected in WT cells transformed with overexpressed Ire1^{LD}-HA in the absence and presence of tunicamycin (Tm).

Next, a β -galactosidase assay was performed to demonstrate that UPR activation can indeed be rescued from the competitive binding of misfolded proteins to the LD. We expect the uncoupling of UPR^{PT} and UPR^{LBS} and the resulting activation of the UPR to be solely that of UPR^{LBS}. The functionality of the LD was confirmed when the UPR response was attenuated in tunicamycin treated cells upon LD overexpression, suggesting it competes with Ire1 to bind misfolded protein ($p= 0.0023$, Figure 24). The small, insignificant UPR activation of cells in the absence of protein stress upon OE Ire1^{LD} in *IRE1 ire1* Δ cells may be due to the non-specific binding of the LD to hydrophobic patches of folding proteins, preventing proteins from achieving their final conformation, creating dysregulated proteostasis and leading to a global cellular response of upregulating protein stress (Figure 24). In the presence of misfolded proteins, the OE Ire1^{LD} in *IRE1 ire1* Δ cells rescued the UPR significantly ($p= 0.0023$), indicating that the luminal domain is sufficient to rescue endogenous Ire1 from tunicamycin induced UPR^{PT} (Figure 24). During UPR^{LBS}, the overexpression of the LD significantly reduces the strength of UPR induction ($p= 0.0114$, Figure 24). Protein stress, in the form of misfolded proteins, cannot be detected by Ire1 due to the competitive binding by the LD, leaving only UPR^{LBS}, which is not sensed by the luminal domain of endogenous Ire1. The results confirm the dispensability of the luminal domain during UPR^{LBS} but not during UPR^{PT}. The data also confirms the cumulative effect of proteotoxic stress accompanying lipid bilayer stress in our PC depleted model. This further explains the stronger induction of the UPR in *IRE1 ire1* Δ *opi3* Δ cells, due to the integration of misfolded protein stress and lipid bilayer stress onto Ire1 (Figure 24). Indeed, relative UPR activity was significantly reduced ($p= 0.0238$) in *Δ LD ire1* Δ *opi3* Δ cells upon the removal of the misfolded protein stress sensing domain, suggesting the efficiency of Ire1 sensing in the presence of the luminal domain (Figure 21). The dual function of the UPR in protein and lipid homeostasis suggests that lipid bilayer stress induces proteotoxicity and these two types of stress are interconnected. Upon the conclusion of this experiment, it was pointed out that the additional conditions of *Δ LD ire1* Δ *opi3* Δ cells and *Δ LD* overexpressing Ire1^{LD} in *ire1* Δ *opi3* Δ cells should have been included in the β -galactosidase assay of Figure 24 to prove the abovementioned hypothesis of an accompanying accumulation of misfolded proteins during UPR^{LBS}. The predicted outcome from the comparison of these two additional strains would have been an indifference in UPR activation. This would have strengthened the conclusion of this experiment.

Figure 24. Overexpression of Ire1 luminal domain (*OE IRE1^{LD}*) rescues the UPR from misfolded protein stress. *ire1*Δ mutants carrying only *IRE1* or *IRE1* and *OE IRE1^{LD}* were left uninduced (no tunicamycin) or induced with 2.5 μg/ml tunicamycin (Tm). *ire1*Δ*opi3*Δ mutants carrying only *IRE1* or *IRE1* and *OE IRE1^{LD}* were left untreated. The strains were assayed for β-galactosidase activity and normalised to untreated *IRE1 ire1*Δ. Error bars represent the standard error mean of three biological replicates.

The Hsp70 family chaperone Kar2 is important for maintaining ER quality control [196]. Kar2 functions as a negative regulator of Ire1 through its association to Ire1 luminal domain and has additional roles in protein translocation and ERAD [30, 197, 198]. Importantly, Kar2 binds excessive misfolded proteins in the ER lumen to cope with unfolded protein stress. We overexpressed Kar2 in *IRE1 ire1*Δ and *IRE1 ire1*Δ*opi3*Δ mutants to determine if the effects of its chaperone function will be similar to the UPR rescue observed with LD overexpression (Figure 25A, 25B). In contrast to the *OE Ire1^{LD}*, the overexpression of Kar2 (*OE KAR2*) does not reduce UPR induction in WT cells subjected to proteotoxic stress (Figure 25C). A similar overexpression of *KAR2* did not relieve cells of ER stress [193]. The lack of rescue can be attributed to differences in substrate specificity between the LD and Kar2 [24, 39]. The abundance of Kar2 in the ER lumen may additionally contribute to a certain degree of heterogeneity of Kar2 levels, making it difficult to achieve significant Kar2 overexpression above basal levels therefore resulting in a lack of UPR rescue [41,199] (Figure 25A, 25B). Another limitation that can be considered is the importance of the BiP/Kar2 ATPase cycle for optimal substrate binding, folding and release [26] (Figure

2). The process of trapping a misfolded protein in the substrate binding domain of Kar2 for correct folding and its subsequent substrate release is highly dependent on the co-chaperone J-domain proteins and NEFs [26]. The lack of UPR rescue observed here may be due to the inability of overexpressed Kar2 to function properly as a chaperone due to the lack of accompanying J-domain proteins and NEFs overexpression. There was no difference in UPR induction with the OE of Kar2 in *IRE1 ire1 Δ opi3 Δ* cells (Figure 25C). The lack of difference observed in *IRE1 ire1 Δ* and *IRE1 ire1 Δ opi3 Δ* cells as well as the UPR increase observed in *Δ LD ire1 Δ opi3 Δ* cells overexpressing Kar2 can be attributed to the untimely release of substrates by Kar2 inhibiting its proper cellular protein functions such as folding as well as translocation and ERAD, thereby resulting in additional ER stress. This also emphasises the substrate specificity of the luminal domain of Ire1 and its ability to directly bind a certain subset of misfolded proteins, which challenges the alternative hypothesis of Ire1 activation upon the release of Kar2 when it binds to misfolded protein during ER stress [30].

The replacement of Ire1 luminal domain by a dimer forming “basic-region leucine zipper” (bZIP) motif retained Ire1’s activity, indicating that the UPR can be switched on by triggers other than misfolded protein stress [64]. Since this activation is independent of the core stress sensing region (CSSR), the dispensability of Ire1 luminal domain in UPR^{PT} was demonstrated [64]. As previously mentioned, crystallisation and mutational studies have shown that Ire1 luminal domain can directly interact with misfolded proteins [39]. Our data suggests that the overexpression of Ire1 luminal domain rescues the UPR observed in *ire1 Δ opi3 Δ* cells, leaving only UPR activation due to an increased PC/PE ratio (Figure 24).

Figure 25. The overexpression of Kar2 (*OE KAR2*) fails to rescue the cells from the UPR during both proteotoxic and lipid bilayer stress. (A) The levels of *OE Kar2* was detected by western blot in *IRE1 ire1Δ* and *IRE1 ire1Δopi3Δ* cells overexpressing Kar2. (B) Quantification of Kar2 levels normalised to tubulin in (A) by ImageJ 1.48v reveals a possible increase in Kar2 levels in *IRE1 ire1Δ* and *IRE1 ire1Δopi3Δ* cells overexpressing Kar2. Results of one technical replicate. (C) *ire1Δ* mutants carrying only *IRE1* or *IRE1* and *OE KAR2* were left uninduced (no tunicamycin) or induced with 2.5 µg/ml of tunicamycin (Tm). *ire1Δopi3Δ* mutants carrying only *IRE1* or only *IRE1ΔLD* or *IRE1* and *OE KAR2* or *IRE1ΔLD* and *OE KAR2* were left untreated. The strains were assayed for β-galactosidase activity and normalised to untreated *ire1Δ* carrying *IRE1*. Error bars represent the standard error mean of three biological replicates.

3.5 Lipid bilayer stress induces Ire1 without oligomerisation

Studies have shown that Ire1 exists as inactive monomers during resting state and form back to back dimers followed by active higher order oligomers during ER stress [46, 200]. In *S. cerevisiae*, *in vivo* evidence of Ire1 clustering upon ER stress was visualised by time-lapse confocal imaging of GFP-Ire1 in response to DTT treatment [129]. We sought to determine the difference in *in vivo* oligomerisation

state of Ire1 during protein stress and lipid bilayer stress by an approach termed as the bimolecular fluorescence complementation (BiFC) method or the Split Venus method which in principle, is based on the reassembly of a Venus protein from its two complementary non-fluorescent fragments upon interaction [201]. This allows us to label Ire1 in living cells and enable the visualisation of *in vivo* real time changes to the labelled protein and its oligomerisation state by confocal live cell imaging. The aim is to determine the clustering state of Ire1 in cells that were exposed to acute proteotoxic stress by DTT treatment and the effects of membrane lipid bilayer stress such as inositol depletion and *OP13* deletion (Figure 26). Complementary halves of Venus were constructed as N-terminally epitope tagged forms of Ire1: Ire1-VN-HA/Ire1-VC-FLAG and Ire1 Δ LD-VN-HA/Ire1 Δ LD-VC-FLAG (simplified as I Δ LD-VN-HA or I Δ LD-VC-FLAG) (Figure 26). Both halves of the Venus constructs were co-expressed into *ire1* Δ and *ire1* Δ *opi3* Δ cells and subject to different conditions of proteotoxic stress and lipid bilayer stress. Split Venus constructs were endogenously expressed Ire1 or I Δ LD to eliminate false activation or clustering of the protein. This ensures that the strength of the oligomerisation state of Ire1 observed reflects the type of stress occurring within the cells.

Figure 26. Split Venus illustration of Ire1/Ire1 Δ LD-VN-HA/VC-FLAG subject to either proteotoxic stress and/or lipid bilayer stress. *ire1* Δ and *ire1* Δ *opi3* Δ cells expressing N-terminally epitope tagged forms of Ire1: Ire1-VN-HA/Ire1-VC-FLAG and Ire1 Δ LD-VN-HA/Ire1 Δ LD-VC-FLAG (simplified as I Δ LD-VN-HA or I Δ LD-VC-FLAG). Ire1-VN and Ire1-VC or I Δ LD-VN and I Δ LD-VC can only form functional fluorescing Venus proteins upon oligomerisation.

For detection of the proper localisation of the Split Venus constructs, indirect immunofluorescence against HA and FLAG was performed. Ire1/ Δ LD-VN-HA and Ire1/ Δ LD-VC-FLAG colocalised with the ER marker Kar2 confirming its localisation to the ER (Figure 27).

Figure 27. The Venus tags do not affect the localisation of Ire1 and Ire1 Δ LD to the ER. Indirect immunofluorescence of *ire1* Δ and *ire1* Δ *opi3* Δ cells expressing Ire1-VN-HA or Δ LD-VN-HA and Ire1-VC-FLAG or Δ LD-VC-FLAG with endogenous Kar2 as an ER marker. Scale bar: 5 μ m. At least 30 cells per condition of randomly chosen fields were imaged for analysis for each replicate. The represented images are of 2-3 cells per condition.

Next, the cells were imaged with confocal live-cell imaging to observe functional fluorescing Venus signal in the event of oligomerisation. As positive control, *ire1* Δ expressing Ire1-VC and Ire1-VN cells were subjected to 10 mM DTT for 1 hour and the formation of puncta was observed within cells (Figure 28A, lane 2; red arrows). As expected, puncta formation was absent in *ire1* Δ cells expressing Ire1 Δ LD-VC and Ire1 Δ LD-VN subjected to DTT (Figure 28A, lane 4). We next looked at another form of lipid bilayer stress known to induce the clustering of Ire1. Previously, observed Ire1 clusters of low intensity formed upon inositol depletion [64, 67, 202]. Inositol depletion causes LBS since it is a precursor of phospholipid biosynthesis. To validate this finding with the Split Venus method, we depleted Ire1-VN and Ire1-VC expressing *ire1* Δ cells of inositol for 4 hours and observed an absence of puncta within these cells (Figure 29, lane 2). Similar to our findings, during LBS by palmitic acid supplementation in mammalian cells, Ire1 α clusters could not be observed by the Split Venus methodology [203]. Fluorescence intensity of the *UPRE-GFP* Ire1 query strain shows that the UPR activation during inositol depletion was similar to that of DTT treated WT cells (Figure 30). Since the oligomerisation of Ire1 is

required for the activation of its cytosolic kinase and RNase domain, we next determined downstream *HAC1* splicing using the Split Venus strains. This is to ensure that the level of *HAC1* splicing corresponds to the upstream oligomerisation status of Ire1. Spliced *HAC1* was observed in inositol depleted Ire1-VN/VC expressing *ire1Δ* cells ($p=0.0273$), indicating that Ire1 is active (Figure 31). This suggests that during LBS by inositol depletion, Ire1 may form active homodimers that correspond to a single Venus protein that although undetectable by confocal microscopy, is important for achieving the RNase splicing competent state for *HAC1* mRNA processing.

In mammalian cells, recent work by quantitative microscopy revealed that overexpressed Ire1 α forms between 10-100 Ire1 α clusters upon UPR^{PT} [204]. These Ire1 α clusters are concomitantly dispersed across the ER network. Upon continual exposure to ER stress, the size of these Ire1 α clusters grow by lateral diffusion and the incorporation of additional free Ire1 α molecules from the surrounding ER network, until a stable plateau is reached [204]. Unabated ER stress eventually attenuates Ire1 α signalling, bringing the plateau phase to an end, resulting in the dispersion of Ire1 α molecules back into the ER network [204]. In *S. cerevisiae*, a model of Ire1 higher order assembly was proposed by Korennynkh *et al.* [205]. This activation model featured a two-dimensional arrangement of Ire1 that involves the incremental addition of symmetric back-to-back Ire1 dimers consisting of approximately 20-30 molecules of Ire1 during UPR^{PT} [205]. This proposed model is consistent with the size of Ire1 foci that was observed *in vivo* by fluorescence microscopy upon DTT treatment [49, 206]. Comparison of the size of the Ire1 foci (DTT treated) observed in our Split Venus experiment, using the same set parameters as previously defined by Halbleib *et al.* suggests a similarity in the size of foci observed (Figure 28, lane 2; red arrows) [67]. This suggest that as published in the abovementioned literature, the size of an observable Ire1 foci observed during protein stress should consist of approximately 20-30 Ire1 molecules whilst during lipid bilayer stress, Ire1 molecules may exist as single homodimers, unobservable by fluorescent microscopy.

Figure 28. Ire1 oligomerisation is not necessary for activation during PC depletion. (A) Split Venus constructs Ire1-VN/Ire1-VC in *ire1Δ* and *ire1Δopi3Δ* cells form puncta upon treatment with 10 mM for 1 hour. ΔLD-VC/ΔLD-VN in *ire1Δ* and *ire1Δopi3Δ* cells did not form puncta upon proteotoxic stress or LBS. The red arrows indicate Ire1 puncta. Scale bar: 5 μm. At least 30 cells per condition of randomly chosen fields were imaged for analysis for each replicate. The represented images are of 2-4 cells per condition. (B) An increase in Ire1 puncta per cell was observed in cells experiencing cumulative UPR^{LBS} and UPR^{PT} stress. Ire1-VN/VC expressing *ire1Δ* and *ire1Δopi3Δ* cells were subjected to 10 mM of DTT and the quantification of high intensity Ire1 clusters per cell was performed using ImageJ 1.48v. Total number of cells analysed for each condition across three biological replicates are 235 Ire1-VN/VC expressing *ire1Δ* cells and 105 Ire1-VN/VC expressing *ire1Δopi3Δ* cells. Error bars represent the standard error mean of three biological replicates.

Figure 29. Ire1 does not form puncta upon inositol depletion. Live confocal imaging of the Split Venus strains Ire1-VN/Ire1-VC or ΔLD-VN/ΔLD-VC expressing *ire1Δ* cells either untreated or depleted of inositol for 4 hours. Cells depleted of inositol were washed and grown in inositol free selective media

for 4 hours. Scale bar: 5 μ m. At least 30 cells per condition of randomly chosen fields were imaged for analysis for each replicate. The represented images are of 2 cells per condition.

Figure 30. Inositol depletion and DTT treatment activates the UPR in Ire1 but not in Ire1 Δ LD. Query strains *Ire1* and *Ire1* Δ LD with genomically integrated *UPREpr-GFP* and *TEF2pr-mCherry* were used to test the effect of inositol starvation on UPR activation. Histogram representing GFP fluorescence of Ire1 and Ire1 Δ LD cells untreated (black), treated with 10 mM of DTT for 1 hour (blue) or depleted of inositol (- inositol) for 4 hours (orange). The values represent the mode of the fluorescent signal.

Like *ire1* Δ cells expressing Ire1-VN/Ire1-VC, we observed a lack of puncta formation and UPR activation in *ire1* Δ expressing Δ LD-VN/ Δ LD-VC cells upon inositol depletion (Figure 29, 30). While *HAC1* splicing during inositol depletion of Δ III Ire1 was shown, there was no evidence of cluster formation as demonstrated by Promlek *et al.* [64]. Contrary to their findings, we did not observe *HAC1* splicing in *ire1* Δ expressing Δ LD-VN/ Δ LD-VC cells (Figure 31). Although inositol is a major precursor of phospholipid biosynthesis, inositol depletion has also been reported to lead to altered ER oxidative folding stress within the ER lumen, possibly explaining that yet another perturbation to ER homeostasis (another stress other than DTT or Tm induced) leads to a difference in cluster formation [207]. Our data suggests that clustering ability of Ire1 and UPR^{LBS} activation by inositol depletion may be dependent on the LD (See Table 8 for a summary of the comparison of Ire1 cluster formation and *HAC1* splicing between Ire1 Δ LD and Δ III Ire1). When the luminal domain of Ire1 was substituted with the bZIP domain of the yeast transcription factor Gcn4, bZIP-Ire1 clustered during both non-stress and inositol depletion conditions [64]. The formation of bZIP-Ire1 clusters in the absence of stress indicates there are other

factors and Ire1 domains involved in driving Ire1 oligomerisation such as the transmembrane domain [66] or the cytosolic domain of Ire1 [205].

We then examined the effect of PC depletion in *ire1Δopi3Δ* cells on oligomerisation. No puncta formation was observed in Ire1-VN/VC expressing *ire1Δopi3Δ* cells although *HAC1* splicing still occurred (Figure 28A, 31). To determine if there was a difference in Ire1 clustering dynamics during UPR^{PT} and UPR^{LBS}, we subjected Ire1-VN/VC expressing *ire1Δopi3Δ* cells to additional acute proteotoxic stress by exposing them to 10 mM DTT for 1 hour. This leads to both the cumulation of proteotoxic and lipid bilayer stress. Surprisingly, puncta forming ability was retained upon DTT treatment of *ire1Δopi3Δ* cells expressing Ire1-VN/VC (Figure 28A, lane 6; red arrows). The quantification of these clusters shows that there is a slight increase ($p=0.0331$) in clusters when UPR^{PT} is combined with UPR^{LBS}, suggesting more Ire1 clusters upon cumulative ER stress (Figure 28B). Altogether, our data suggests that differences in the degree of ER stress (DTT treatment *versus* inositol depletion) on Ire1 affects its clustering ability. In addition, DTT treatment of *opi3Δ* cells suggests that higher order Ire1 clustering during LBS is highly dependent on the luminal domain. This is likely due to misfolded proteins acting as a driving force for the oligomerisation of the luminal domain [24]. Our Split Venus experiment revealed that Ire1 clusters increase in response to DTT induced ER stress but is absent during inositol depletion and *opi3Δ* induced lipid bilayer stress. We found that whereas there was no UPR activation and *HAC1* splicing observed for Δ LD-VN/VC cells during inositol depletion, *HAC1* splicing was observed in the *ire1Δopi3Δ* cells expressing Δ LD-VN/VC (Figure 30, 31). Our data suggests a concerted mechanism of oligomerisation formation involving the luminal domain directly and possibly the recruitment of other yet defined molecules as a driving force in response to different stressors.

Our data, together with the literature, suggest varying differences in Ire1 clustering and activation dynamics during UPR^{PT} and UPR^{LBS}, caused by inositol depletion or *OPI3* deletion. Time course changes in cellular *HAC1* splicing demonstrated that the activation of Ire1 to DTT was quick and robust (peak activation within 30 min to 1 hour of stimulus) while *HAC1* splicing occurs at later time-points during inositol depletion in Ire1, Δ III Ire1 and bZIP-Ire1 cells [64]. This may be due to time required for the depletion of free inositol within the cell or indicates a milder, more chronic form of ER stress. While we did not perform time course experiments of *HAC1* splicing in the *opi3Δ* model of LBS, the constitutive activation of the UPR observed in *opi3Δ* cells similarly suggests chronic ER stress during LBS with differential Ire1 activation dynamics compared to UPR^{PT}.

Figure 31. *HAC1* mRNA is spliced during inositol depletion in Ire1 but not Ire1 Δ LD expressing cells. (A) *ire1Δ* mutants carrying *IRE1-VN/VC* and *ΔLD-VN/VC* were left uninduced (no DTT), induced (10 mM DTT) or inositol depleted (inositol) and *ire1Δopi3Δ* mutants carrying *IRE1-VN/VC* and *ΔLD-VN/VC* were left untreated (no choline) or treated with 1 mM choline (cho). *HAC1* mRNA was detected by RT-PCR. The two forms of *HAC1* are indicated as *HAC1^u* (unspliced) and *HAC1^s* (spliced). (B) Quantification of the *HAC1^s* over total *HAC1* in (A) by ImageJ 1.48v confirms *HAC1* splicing in *IRE1-VN/VC ire1Δ* cells during inositol depletion that is absent in *ΔLD-VN/VC ire1Δ* cells. Error bars represent the standard error mean of three biological replicates.

The commonality of Δ LD, Δ III Ire1 and bZIP-Ire1 are their transmembrane domains (TM), combined with the evidence that Δ LD-Ire1 α was activated upon lipid bilayer stress due to membrane saturation and that the activation was crucial on the tethering of the TM to the ER membrane prompted us to further dissect the stress sensing function of the TM, described in the next section [66, 83].

3.6 A key arginine residue is essential for UPR^{LBS}

Membrane aberrancy due to either inositol depletion or saturated fatty acid enrichment activates the UPR [64, 81]. In mammalian cells, saturation of the ER lipid bilayer triggers a response that causes the activation and subsequently oligomerises Ire1 α independently of their respective luminal domain [66, 83]. Data show that the tethering of the transmembrane (TM) domain to the ER membrane is essential for Ire1 α activation *in vitro*, suggesting its role as a direct lipid sensor to change in membrane saturation

[66]. Recently, it was shown that a serine 450 residue and tryptophan 457 residue of the Ire1 α TM domain senses membrane saturation independently of the luminal domain and dimerises *in vivo* [83] (Figure 32, pink). We attempted to identify potential lipid bilayer stress sensing residues in the membrane spanning region of Ire1.

As the luminal domain is dispensable in sensing UPR^{LBS} in *opi3 Δ* cells, the TM domain is likely to sense changes in phospholipid composition. In yeast, the TM domain of Ire1 is predicted to be a 29 amino acid domain consisting of only two charged amino acids, the rest being hydrophobic amino acids with aliphatic, neutral and aromatic side chains. The TM domain of Ire1 consists of a putative amphipathic helix (that overlaps on the ER luminal side) and a transmembrane helix [67] (Figure 32). Recently, the juxta-membrane amphipathic helix of Ire1 was shown to be required for sensing both proteotoxic stress and LBS [67]. Molecular dynamics simulations and in-vitro assays show that the amphipathic helix at the juxta-membrane region of Ire1 forms a kink in the transmembrane domain, causing local membrane compression [67]. The energetic cost of this local membrane compression increases upon increased membrane thickness and lipid packing during lipid bilayer stress, which causes Ire1 to form energetically favourable dimers that is stabilised by its surrounding membrane environment. The residues responsible for the function of the amphipathic helix of Ire1 during both proteotoxic stress and lipid bilayer stress is represented by the F531R and V535R mutations (Figure 32, green). Despite its high homology in mammalian cells, mutation of the amphipathic helix residue V437R and M440R did not affect the dimerization and downstream *XBP1* mRNA splicing in Ire1 α - Δ LD cells (Figure 32, orange). Instead, activation of Ire1 α was highly dependent on the serine 450 and tryptophan 457 residues in response to palmitate loading, confirming the role of the transmembrane domain in lipid sensing (Figure 32, pink). We sought to uncouple UPR^{PT} from UPR^{LBS} and identify amino acid residues within the transmembrane domain sensitive to only lipid bilayer stress. Since the crystal structure of Ire1 transmembrane domain has not been determined, we relied on bioinformatics prediction that revealed two oppositely charged residues, R537 and E540, in close proximity to one another within the α -helix (Figure 32, purple). We hypothesized that the electrostatic attraction between these two residues acts as the driving force for UPR activation during lipid bilayer stress. In support of this data, we have identified that the positively charged residue arginine 537 governs the responsiveness of Ire1 only to lipid bilayer stress, and not to Tm induced ER stress, reinforcing the role of the amphipathic helix as a lipid sensor (Figure 32, purple).

Figure 32. Ire1 arginine residue at position 537 sits in amphipathic helix and at the juxta-region of the transmembrane helix. The arginine (R) at position 537 (purple) was mutated to the neutral residue glutamine (Q) to determine the effect of the point mutation on lipid bilayer stress. Other compromising mutations are the F531R and V535R mutations (green) in the amphipathic helix of Ire1 and the S450A and W457A (pink) residues in the transmembrane helix of Ire1α that affected Ire1α/Ire1 activity during lipid bilayer stress [67, 83]. The mutation of conserved residues in the amphipathic helix of Ire1α V437R and M440R (orange) and L441R did not affect the activity of Ire1α during lipid bilayer stress [83].

The residue R537 was mutated to a non-charged glutamine (Q) residue and indirect immunofluorescence experiment was performed to ensure that the introduction of the mutation does not mis-localise the protein. Indirect IF images show that Ire1(R537Q) and Ire1ΔLD(R537Q) (simplified as IΔLD(R537Q)) cells in both *ire1*Δ and *ire1*Δ*opi3*Δ cells localise correctly to the ER as observed by its localisation with ER marker Kar2 (Figure 33).

A spotting assay was performed to determine the functionality of Ire1(R537Q) or IΔLD(R537Q) during proteotoxic and lipid bilayer stress (Figure 34). Cells were spotted onto selective media and no defects in cell growth were observed in the presence of the R537Q mutations when comparing *IRE1 ire1*Δ to *IRE1(R537Q) ire1*Δ cells as well as *IΔLD ire1*Δ to *IΔLD(R537Q) ire1*Δ cells (Figure 34). Cells were spotted onto tunicamycin plates as a measure of fitness to observe the effect of the R537Q mutation during proteotoxic stress (Figure 34). Other than the slight delay in growth due to the stress of tunicamycin, *IRE1(R537Q)* and *IΔLD(R537Q) ire1*Δ cells exhibited no growth defects as compared to their non-mutated counterparts (Figure 34). Similarly, in *IRE1* and *IRE1(R537Q) ire1*Δ*opi3*Δ cells as well as *IΔLD* and *IΔLD(R537Q) ire1*Δ*opi3*Δ cells, the mutation had no effect on growth of the strains. The effect of *opi3*Δ mutation could be restored by spotting these cells on choline supplemented selection plates (Figure 34).

Figure 33. Proper integration of Ire1(R537Q) and Ire1ΔLD(R537Q) by indirect immunofluorescence. Indirect immunofluorescence of *ire1Δ* and *ire1Δopi3Δ* mutants expressing N-terminally Flag tagged Ire1(R537Q) and IΔLD(R537Q) with endogenous Kar2 as an ER marker. Scale bar: 5 μm. At least 30 cells per condition of randomly chosen fields were imaged for analysis for each replicate. The represented images are of 3-4 cells per condition.

Figure 34. Ire1(R537Q) mutant is functional during UPR^{PT} and UPR^{LBS}. Ire1 lacking its luminal domain is sufficient to rescue cells from LBS. *ire1Δ* and *ire1Δopi3Δ* mutants carrying *IRE1*/*IRE1(R537Q)* and *IΔLD*/*IΔLD(R537Q)* were grown in synthetic complete selective medium, diluted to 0.2 OD cells, serially diluted 1:10 and spotted onto uninduced no tunicamycin, 0.125 μg/ml tunicamycin and 1 mM choline plates as indicated. Plates were incubated at 30°C for 2 days.

Since there were no obvious defects on cell growth, we looked at the effect of the R537Q mutation on downstream *HAC1* mRNA splicing and subsequently quantified the amount of spliced *HAC1* (Figure 35, 36). There was no significant difference in *HAC1* mRNA splicing when comparing *IRE1* to *IRE1(R537Q) ire1Δ* cells and *ΔLD* to *ΔLD(R537Q) ire1Δ* cells subject to tunicamycin (Figure 35, 36). *HAC1* mRNA was spliced to a similar extent in *IRE1 ire1Δ* and *IRE1(R537Q) ire1Δ* cells (Figure 35, 36). As expected, the R537Q mutation had no effect on *HAC1* splicing in *ΔLD(R537Q) ire1Δ* compared to *ΔLD ire1Δ* cells (Figure 35). Interestingly, in *ire1Δopi3Δ* cells, R537Q virtually abolished *HAC1* mRNA splicing in both *IRE1* and *ΔLD* constructs (Figure 35, 36). *HAC1* mRNA splicing was significantly higher in *IRE1* and *ΔLD ire1Δopi3Δ* cells which was eliminated by the R537Q mutation (Figure 35, 36). Cells are dependent on UPR activation for survival upon the deletion of *OPI3*. The lack of *HAC1* splicing (Figure 35, 36) observed in *IRE1(R537Q)* or *ΔLD(R537Q) opi3Δ* cells suggests an undetectable level of *HAC1* splicing (by RT-PCR) that ensures cell viability. Alternatively, it is possible that other Ire1 independent mechanisms are initiated as an adaptive response to compensate for the decrease in *HAC1* splicing required for cell survival (this is further discussed in section 4.2). Our data suggest the potential of this residue in distinguishing between UPR^{LBS} and UPR^{PT}. The activation of the UPR is rescued with the supplementation of choline regardless of the presence of the mutation (Figure 35, 36).

Figure 35. The residue arginine 537 of Ire1 is essential for lipid bilayer stress sensing. Cells were left uninduced or induced with 2.5 μg/ml of tunicamycin (Tm) and untreated (no choline) or treated 1 mM choline (cho) overnight, as indicated. *HAC1* mRNA was detected by RT-PCR. The two forms of *HAC1* are indicated as *HAC1*^U (unspliced) and *HAC1*^S (spliced).

Figure 36. *HAC1* mRNA splicing under proteotoxic and lipid bilayer stress with and without the R537Q mutation. The R537Q mutation has no effect on cells during proteotoxic cells but completely attenuates *HAC1* splicing during lipid bilayer stress. *ire1Δ* mutants carrying *IRE1* or *IRE1* (R537Q) and *IΔLD* or *IΔLD* (R537Q) were left uninduced (no tunicamycin) or induced with 2.5 μg/ml tunicamycin (Tm). *ire1Δopi3Δ* mutants carrying *IRE1* or *IRE1* (R537Q) and *IΔLD* or *IΔLD* (R537Q) were left untreated or treated with 1 mM choline overnight (cho). The strains were assayed and normalised to actin. The values represent *HAC1* spliced over total *HAC1*. Error bars represent the standard error mean of three biological replicates.

Since *HAC1* mRNA splicing is abolished in the presence of LBS with the R537Q mutation, we looked downstream to determine if Hac1 target gene *KAR2* levels are affected [208]. Indeed, *KAR2* mRNA levels were upregulated during proteotoxic stress indicating positive regulation by the activation of the UPR (Figure 37). A similar observation was previously reported by Hsu *et al.* that the expression of Kar2 protein increased during tunicamycin treatment of wild-type *S. cerevisiae* cells [196]. The levels of *KAR2* was significantly downregulated in *IRE1*(R537Q) and *IΔLD*(R537Q) *ire1Δopi3Δ* cells confirming the consequence of the lack of *HAC1* mRNA splicing observed above (Figure 35, 37). This suggests that R537 is necessary for LBS sensing, the mutation of which results in a lack of *HAC1* splicing and downstream target gene upregulation.

Figure 37. The absence of *HAC1* splicing correlates with *KAR2* mRNA expression levels. Relative *KAR2* mRNA levels are downregulated in the presence of the R537Q mutation in both Ire1 and Ire1ΔLD during lipid bilayer stress. *ire1Δ* mutants carrying *IRE1* or *IRE1* (R537Q) and *IΔLD* or *IΔLD* (R537Q) were left uninduced (no tunicamycin) or induced with 2.5 μg/ml of tunicamycin (Tm). *ire1Δopi3Δ* mutants carrying *IRE1* or *IRE1* (R537Q) and *IΔLD* or *IΔLD* (R537Q) were left untreated or treated with 1 mM choline (cho). The strains were assayed and normalised to *ire1Δ* cells expressing Ire1. Error bars represent the standard error mean of three biological replicates.

3.7 Ire1 employs a divergent transcriptional programme in response to lipid bilayer stress

The UPR activates a broad-spectrum compensatory response when cells are inflicted with stress. The type of stress determines the strength of the UPR and targets differential gene expression for the compensatory response [132]. The identification of 381 UPR target genes by microarray hybridisation expression profiling revealed the importance of proteins involved in the ER, Golgi apparatus and secretory pathway in remodelling the cellular landscape of the cell during ER stress [50]. The presence of the *UPRE-1*, characterised as a Hac1 binding motif, was however not present in all 381 UPR target genes previously identified [162]. This finding was surprising since Hac1 was thought to be required for the activation of all UPR target genes. This led to the speculation of the presence of other *UPRE* motifs or trans-acting factors that may act alone or in combination with Hac1 and contribute to the transcriptional complexity of the UPR [162]. Indeed, two other *UPRE* motifs: *UPRE-2* and *UPRE-3* were

identified and the yeast transcription factor Gcn4, a regulator of amino acid biosynthesis was discovered as a modulator of Hac1 activity [162]. Gcn4 was found to be transiently upregulated in the beginning of UPR induction, indicating an increase in transcriptional output of the UPR [162]. Since Gcn4 acts independently as a transcription factor and upregulates its own subset of genes, the recruitment of Gcn4 to Hac1 is evidence of a distinct response involving crosstalk between regulatory pathways and the fine-tuning of the UPR response. Furthermore, a combination of DTT-induced protein stress and heat stress led to the induction of differential UPR target genes compared to DTT-induced protein stress alone [209]. DNA microarray chip analysis identified three classes of genes that were categorised according to fold induction [209]. Interestingly, *OPI3* belonged to class 2 of these genes where they observed a 2-4-fold gene expression induction in response to protein and heat stress [209]. This led us to consider the possibility of an alternative transcriptional programme that could be mounted in response to LBS by an alternate stress in the form of PC depletion. Identification of the genes activated by UPR^{LBS} that are unique from genes upregulated by UPR^{PT} will shed light on the additional mechanisms or proteins involved in restoring cellular balance during LBS. DNA microarray was performed in *IRE1* or *IRE1ΔLD ire1Δ* and *ire1Δopi3Δ* cells to identify the differential gene expression profile during LBS (Figure 38). Total RNA was extracted from *IRE1* or *IRE1ΔLD ire1Δ* and *ire1Δopi3Δ* cells untreated or treated with either DTT (to induce UPR^{PT}) or supplemented with choline (to rescue UPR^{LBS} in *opi3Δ* cells) (Figure 38). Before determining the genes upregulated from UPR^{PT} and UPR^{LBS}, we first normalised each transcriptomic data set to *IRE1 ire1Δ* cells (Figure 38B). Since *ire1Δopi3Δ* cells could not be included in our control analysis due to synthetic lethality, gene targets of UPR^{LBS} were further filtered by excluding previously identified UPR^{PT} target genes [50]. A gene was selected as upregulated when expression is more than 2-fold increased. The overlap of genes upregulated by DTT treatment in *IRE1* and *IRE1ΔLD ire1Δ* cells and by *OPI3* deletion in *IRE1* and *IRE1ΔLD ire1Δopi3Δ* cells are compared and represented below in Figure 39. Functional classification of genes represented in Figure 40 was performed using gene ontology tool DAVID.

Figure 38: Schematic outline of the DNA microarray performed to identify differences in transcriptomic profiles of genes during UPR^{PT} and UPR^{LBS}. (A) Total RNA is first isolated from *IRE1* or *IRE1ΔLD* expressing *ire1Δ* and *ire1Δopi3Δ* cells untreated or treated with either 10 mM DTT, 1 hour (to induce UPR^{PT}) or grown in media supplemented with 1 mM choline (to alleviate UPR^{LBS} in *opi3Δ* cells). RNA extraction is followed by RNA clean-up as a quality control step. The Low Input Quick Amp Labelling System was used for the fluorescent labelling of cDNA during reverse transcription. A custom microarray Agilent GE 8 x 60 K array was generated, probes are hybridised on the custom generated array and scanned using a high-resolution DNA microarray scanner (Agilent Technologies). (B) Analysis of upregulated genes begins by the normalisation of transcriptomic data sets to *IRE1 ire1Δ* cells. The resultant dataset of upregulated genes is further compared and analysed by GO term analysis using the bioinformatics tool DAVID. Upregulated genes are represented in the form of a heatmap, generated by R studio in Figure 42. For detailed methods, see Section 2.14.

Figure 39. Genes upregulated in different subsets of proteotoxic and/or lipid bilayer stress expressing Ire1 and/or Ire1ΔLD. In the overlap of *IRE1* and *IRE1ΔLD ire1Δopi3Δ* cells, 214 (red) genes are positively upregulated. In *IRE1 ire1Δopi3Δ* cells and *IRE1ΔLD ire1Δopi3Δ* cells, 92 (orange) or 56 (blue) genes are upregulated respectively. The overlap of DTT treated *IRE1 ire1Δ* cells and *IRE1* and *IRE1ΔLD ire1Δopi3Δ* cells show an upregulation of 77 (purple) genes, encompassing known gene targets of the UPR. The overlap of DTT treated *IRE1 ire1Δ* cells and *IRE1 ire1Δopi3Δ* cells show an upregulation of 73 (green) genes in response to proteotoxic stress and lipid bilayer stress. See Table S4 in Appendix for the list of genes represented by each of the coloured lanes.

Transcriptome analysis revealed genes that were upregulated by UPR^{PT} and UPR^{LBS}. In response to DTT treatment, 264 genes were upregulated in *IRE1* and *IRE1ΔLD ire1Δ* cells compared to unstressed wild-type cells (Figure 39). Further functional classification of upregulated genes by the gene ontology tool DAVID shows that in response to protein stress and lipid bilayer stress in the overlapping subset of DTT treated *IRE1 ire1Δ* cells and *IRE1* and *IRE1ΔLD ire1Δopi3Δ* cells, 77 genes are upregulated (Figure 39). These genes mount a response to stress pathways, a known gene target of which is the ER integral membrane protein *DER1*, validating the accuracy of the response of target genes [209, 210] (Figure 39, 40, purple). The overlap of 73 genes between DTT treated *IRE1 ire1Δ* cells and *IRE1 ire1Δopi3Δ* cells indicate a convergent upregulation of these genes in response to proteotoxic stress and lipid bilayer stress (Figure 39, green). The GO classification of other gene subsets shows that *IRE1* or *IRE1ΔLD* expressing *ire1Δopi3Δ* cells upregulate a subset of 92 and 56 genes respectively (Figure 39, orange, blue). The weaker induction of the UPR^{LBS} in *IREΔLD ire1Δopi3Δ* cells could also account for the smaller number of genes activated. Our data show that processes such as cellular amino acid metabolism, protein ubiquitination and ER processes are positively upregulated as part of the buffering mechanism in response to lipid bilayer stress (Figure 40, orange, blue).

Figure 40. Gene ontology classification of genes upregulated in conditions of proteotoxic stress and/or lipid bilayer stress in either the *IRE1* or *IRE1*Δ*LD* cells. Functional classification reveal processes enrich in the genes upregulated from *IRE1*Δ*LD* *ire1*Δ*opi3*Δ cells (*IRE1*Δ*LD**opi3*Δ, blue), an overlap of *IRE1* and *IRE1*Δ*LD* *ire1*Δ*opi3*Δ cells (*IRE1**opi3*Δ ∩ *IRE1*Δ*LD**opi3*Δ, red), *IRE1* *ire1*Δ*opi3*Δ cells (*IRE1**opi3*Δ, orange), an overlap of DTT treated *IRE1* Δ*ire1* cells and *IRE1* and *IRE1*Δ*LD* *ire1*Δ*opi3*Δ cells (*IRE1*(DTT) ∩ *IRE1**opi3*Δ ∩ *IRE1*Δ*LD**opi3*Δ, purple) and an overlap of DTT treated *IRE1* *ire1*Δ cells and *IRE1* *ire1*Δ*opi3*Δ cells (*IRE1*(DTT) ∩ *IRE1**opi3*Δ, green). See Table S4 in Appendix for the list of genes represented by each of the coloured lanes.

Remodelling of the proteome and UPR activation occurs in *opi3*Δ mutants upregulating a total of 214 genes (Figure 39, red). These 214 genes include processes such as transmembrane and glucose transport, aerobic respiration and mitophagy (Figure 40, red). Interesting genes involved in mitophagy such as *ATG11* and *ATG32* are required for achieving cellular homeostasis during the UPR [211-213]. A subset of genes within these 214 genes are genes that are likely to be upregulated independently of

the UPR as previously described [65, 214]. These genes could not be excluded as *ire1Δopi3Δ* cells are synthetic lethal because *opi3Δ* is dependent on the UPR for its survival [65]. Since we could not exclude these genes, we filtered gene targets of UPR^{LBS} by excluding previously identified UPR^{PT} target genes leaving 148 genes that were activated uniquely by LBS (Figure 42) [50]. These 148 genes likely contribute to a divergent transcriptional response required for a homeostatic response to lipid bilayer stress (Figure 39, 40, orange and blue).

3.8 Hac1 binds to a differential subset of genes during UPR^{LBS}

To better understand how the global cellular effect of lipid bilayer stress is modulated by the UPR, we looked further into the 148 positively upregulated genes in the *IRE1* and *IRE1ΔLD ire1Δopi3Δ* cells. Since Ire1 is highly dependent on Hac1 to initiate an adaptive response, we attempted to identify Hac1 targets within these genes. Based on the three known *UPRE* motifs Hac1 associates to, we performed bioinformatics analysis to predict for *UPRE* consensus sequence in the promoter of these differentially regulated genes [191]. Bioinformatic analysis revealed 6 genes containing predicted *UPRE*-1, *UPRE*-2 and *UPRE*-3 motifs in their promoters (Figure 41).

<i>KAR2</i>	-160	CAGCGTG	<i>UPRE</i> -1
<i>ACA1</i>	-520	CAGCTTT	<i>UPRE</i> -1
	-663	AGGACATC	<i>UPRE</i> -3
<i>GAC1</i>	-449	TACGTG	<i>UPRE</i> -2
<i>GPH1</i>	-563	CAGCGGG	<i>UPRE</i> -1
<i>PBI2</i>	-280	TACGTG	<i>UPRE</i> -2
<i>PIR3</i>	-510	TACGGG	<i>UPRE</i> -2
<i>PUT1</i>	-799	AGGACACA	<i>UPRE</i> -3
	-823	CAGCGCC	<i>UPRE</i> -1

Figure 41. Predicted Hac1 *UPRE* motifs. The fully defined *UPRE*-1 motif of Kar2 is included as a control. Hac1 preferentially recognises three different *UPRE* binding motifs (*UPRE*-1, *UPRE*-2 and *UPRE*-3) in the promoter of its target genes.

Of the 6 genes identified, one encodes for stress and defence (*PBI2*), which is expected due to the initiation of a stress response. In addition, genes for carbohydrate metabolism (*ACA1*, *GAC1* and *GPH1*), amino acid metabolism (*PUT1*) and cell wall synthesis (*PIR3*) are upregulated (Figure 42;

highlighted in green). These results suggest that Hac1 targets during UPR^{LBS} activates metabolic processes (amino acid and carbohydrate metabolism) for the restoration of cellular homeostasis. Similar to UPR activation induced by ER stress or *opi3Δ*, genes maintaining cell wall integrity were upregulated, suggesting that cell wall stress occurs during UPR activation and the importance of the coordination between cell wall integrity and these cellular responses [50, 65, 215].

Figure 42. Identification of the transcriptional targets during UPR^{LBS}. *ire1Δ* and *ire1Δopi3Δ* mutants expressing Ire1 and Δ LD experienced protein stress (DTT treatment, 1 hour) and lipid stress respectively. The average of the log₂ fold change was calculated for the genes. Only genes with two-

fold differential regulation were considered. Differentially regulated genes were functionally categorised based on gene ontology. Bioinformatics analysis determined 6 upregulated genes containing UPRE motifs in their promoter (highlighted in green).

Due to the high homology of sequences within the promoters to *UPRE-2* and *UPRE-3* motifs, Hac1 may preferentially associate to these 6 target genes for gene upregulation. We first show that HA-Hac1 can indeed be pulled down with a simple immunoprecipitation experiment using the exact lysis and pull-down conditions used for the chromatin immunoprecipitation (ChIP) experiment (Figure 43). To validate our approach, we induced the UPR by tunicamycin treatment and immunoprecipitated HA-Hac1 (Figure 43). The HA-Hac1 band was absent in the empty vector and non-tunicamycin treated controls indicating specificity of the pulldown (Figure 43).

Figure 43. Pulldown of HA-Hac1 during protein stress. *ire1Δ* expressing HA-Hac1 was transformed with an empty vector or *IRE1* and left un-induced or induced with 2.5 µg/ml of tunicamycin. Only Ire1 expressing cells could splice HA-Hac1 in the presence of protein stress. Strains were lysed in a 1% Triton X-100 lysis buffer and immunoprecipitated. The pulldown was performed with a 1:500 dilution of α-HA antibody (Covance) and probed with 1:2500 of α-HA antibody (Covance).

We next performed ChIP in *ire1Δ* and *ire1Δopi3Δ* expressing Ire1 and HA-Hac1 cells to confirm the binding of Hac1 to the *UPRE* motifs of these genes. Immunoprecipitation specific to HA-Hac1 was used to pulldown associating genes in the presence of protein stress. Primers were designed approximately 50 bases upstream and downstream of the *UPRE* for quantitative analysis of these genes. We used *KAR2*, a known Hac1 target containing a *UPRE-1* motif, as a positive control to validate the ChIP experiment and the association of Hac1 to this gene (Figure 44). As expected, *KAR2* levels were

enriched due to Hac1 binding during tunicamycin induced proteotoxic stress and lipid bilayer stress (Figure 44).

Figure 44. Hac1 binds to its UPR target gene *KAR2* during proteotoxic and lipid bilayer stress. HA-Hac1 and Ire1 expressing *ire1Δ* were left untreated (control) or treated with 2.5 μ g/ml tunicamycin (Tm) for 1 hour at 30 °C. HA-Hac1 and Ire1 expressing *ire1Δopi3Δ* cells are left untreated or treated with 1 mM choline (cho) overnight. Error bar represents the standard error mean of three biological replicates

During protein stress, ChIP-qPCR data confirms the absence of fold enrichment of five genes: *PUT1*, *GPH1*, *PBI2*, *PIR3* and *GAC1*, similar to untreated cells (Figure 45). Surprisingly, *ACA1* was significantly enriched in both tunicamycin treated cells and lipid bilayer stress (Figure 45). ChIP-qPCR experiments confirm that 3 genes *PBI2*, *PUT1* and *PIR3* were enriched by Hac1 binding upon lipid bilayer stress (Figure 45). The association of Hac1 to these genes were specific to PC-depleted lipid bilayer stress since gene enrichment was absent when choline was present in the growth media (Figure 45). Although previously identified to be a UPR target gene, *PBI2* was not upregulated in our microarray data during DTT treatment [185]. It is possible that *PBI2* upregulation by DTT yielded a lower response that left it below the threshold of detection. *PBI2* has identified to be upregulated by exogenous inositol. Surprisingly, this gene was not found to be upregulated in the presence of exogenous choline suggesting that *PBI2* is a gene required for buffering changes in lipid homeostasis of the cell [216]. *PUT1* is a proline dehydrogenase required for converting proline to glutamate. Although *PUT1* mutants are relatively insensitive to UPR^{PT}, the upregulation of this gene during UPR^{LBS} suggest the importance

of proline catabolism in restoring lipid homeostasis [217]. The importance of cell wall integrity during UPR^{PT} has previously been reported, therefore it was not surprising that the *PIR3*, a cell wall mannoprotein, although previously unreported, is required for cell wall homeostasis during UPR^{LBS}. This data presented here suggests that *PIR3*, *PBI2* and *PUT1* are novel genes containing UPRE motifs in their promoter that *Hac1* associates to for their upregulation during lipid bilayer stress as part of the homeostatic programme.

Figure 45. *Hac1* binds to *PBI2*, *PIR3* and *PUT1* during UPR^{LBS}. *ire1Δ* mutants expressing HA-*Hac1* and *Ire1* were left uninduced (no tunicamycin) or induced with 2.5 µg/ml tunicamycin (Tm). *ire1Δopi3Δ* mutants expressing HA-*Hac1* and *Ire1* were left untreated or supplemented with 1 mM choline (cho). These cells are subsequently formaldehyde cross-linked and immunoprecipitated with α-HA (1:500). qPCR was performed to quantify the amount of DNA pulled down with HA-*Hac1*. Primers were designed 50 bases upstream and downstream of the UPRE motifs, producing a product of approximately 100 bases. Error bar represents the standard error mean of three biological replicates. The log₂ fold enrichment of each gene was normalised to vector controls. Student's t test, one tailed was used to determine significance.

Chapter 4. Discussion

4.1 Ire1 activation during lipid bilayer stress

Our high throughput genetic screen revealed the importance of certain biological processes required for buffering UPR^{LBS} in *S. cerevisiae*. These genes appear to modulate the UPR, independently of the luminal domain of Ire1 and include processes such as protein glycosylation and mannosylation, ERAD, multiple steps of vesicular trafficking and lipid metabolism, associated to ER membrane integrity. The importance of proper ER membrane integrity has been previously implicated in the UPR. ER morphological changes occur upon the activation of the UPR due to lipid bilayer stress [65]. It was shown that the permeability of the ER membrane is disrupted during lipid bilayer stress, affecting the internal calcium environment and thus the calnexin-calreticulin cycle, activating the UPR [121]. In this thesis, we identified membrane associated networks, such as the vesicular trafficking pathway, that may contribute to ER membrane homeostasis and fine-tune the UPR during lipid bilayer stress. The genes involved in buffering UPR^{LBS} changes indicate the broad impact and interconnectivity of Ire1 in the cell, linking together processes in the global genetic landscape of the cell that are linked to the ER, providing a direct link to Ire1 suggesting a bias in functional relationship to restore homeostatic balance to the cell. Amongst our strongest hits was *OPI3*, a methyltransferase required for PC synthesis that has been implicated in metabolic diseases [121, 218-223]. *OPI3* deletion results in UPR activation caused by a shift in the ER membrane PC/PE ratio which warranted a deeper investigation into the activation mechanism of Ire1 in this model of lipid bilayer stress. It was similarly observed in *Pemt* (homolog of *OPI3*) knockout mice that a decrease in PC/PE ratio led to the perturbation of ER membrane integrity and the progression of steatosis to NASH in the liver [118]. *OPI3* deletion was previously found to remodel the protein homeostasis network [65], therefore it is not surprising that the *OPI3* deletion in our genetic screen activated the UPR. This is further supported by the interdependency of *opi3Δ* cells and the UPR [65]. The activation of UPR^{LBS} in *IRE1* and *ΔLD opi3Δ* cells suggests Ire1 has a secondary function as a sensor responsive to ER membrane composition changes. This mode of Ire1 sensing is different from the better characterised sensing of Ire1 via its luminal domain [39, 42]. While the luminal domain is essential for UPR activation in the presence of misfolded proteins, it is otherwise unimportant in sensing the shift in PC/PE ratio. What was striking to us was the strength of UPR induction upon *OPI3* deletion in both *IRE1* and *ΔLD* cells, suggesting a more efficient mode of

LBS sensing in the presence of the luminal domain. Indeed, cooperativity between adjacent Ire1 domains have been previously described [200, 205]. Binding of misfolded proteins to the luminal domain of Ire1 leads to a conformational change in adjacent domains to initiate autophosphorylation in trans and subsequently, the ribonuclease activity of the protein [205]. Since Ire1 is known to undergo conformational changes to exert its effect, it may be likely that the luminal and transmembrane domain further stabilises Ire1 to enhance the effect of *HAC1* splicing or the efficiency of the signalling during lipid bilayer stress. The difference in strength of UPR induction also suggests that the broad scope of the UPR targets differential subset of genes to attain homeostasis when subjected to different stresses (protein stress *versus* LBS).

Our Ire1^{LD} overexpression experiment suggests that misfolded proteins accumulate during lipid bilayer stress, indicating the relationship between proteotoxicity and LBS. A chemical chaperone 4-phenylbutyrate (4-PBA) was previously demonstrated to prevent protein aggregation *in vivo* [224]. The treatment with 4-PBA alleviated UPR^{LBS}, hinting that proteotoxic stress is a consequence of lipotoxicity [91]. We have previously reported that membrane associated proteins, such as members of the translocon, are destabilised during lipid bilayer stress [225], possibly leading to an interference of protein translocation that could directly cause UPR induction. In *C. elegans*, ablation of *mdt-15*, a regulator of lipid biosynthetic genes, leads to the activation of both IRE-1 and PEK-1 branches of the UPR [193]. Interestingly, in this instance, the activation of both UPR sensors was, however, found to be independent of proteotoxic stress [193]. Because lipid bilayer stress caused by *mdt-15* is rather mild, this suggests the existence of a tipping point, beyond which any present lipid imbalance begins to exert additional burden on the proteostasis network. In either case however, the UPR is activated to cope with changes in protein and/or lipid homeostasis.

4.2 The complexity of the UPR and a regulatory role of the luminal domain of Ire1

Our data hints at the complexity of the UPR. In our genetic screen, more gene deletions activated the UPR in *IRE1ΔLD* cells than *IRE1* cells. This was contrary to what we expected since Ire1 senses ER stress in a luminal domain dependent and independent manner while Ire1ΔLD senses ER stress only in a luminal domain independent manner. In addition, in our spotting assay, *ΔLD ire1Δ* cells were viable. These evidences hint at Ire1-independent mechanisms initiated by the cells to cope with UPR^{PT}.

While the UPR^{LBS} pathway explored in this thesis is Hac1-dependent, there have been reports of additional Ire1-independent pathways in yeast during UPR^{PT}. Colonies of *ire1Δ* and *hac1Δ* strains containing a UPR-*LacZ* reporter turned a light blue colour after prolonged incubation in the presence of tunicamycin [226]. Similarly, with a DNA microarray screen, Travers *et al.* reported that 87 ORFs were induced at least two-fold by ER stress independently of *IRE1* and *HAC1* [50]. Comparison of our genetic screen data to UPR up-regulators in *hac1Δ* cells revealed that the deletion of *IES4* (*Ino-Eighty Subunit 4*), coding a component of the INO80 chromatin remodelling complex [227], upregulates the UPR in *IRE1ΔLD* cells [63]. It is therefore very likely that there are Ire1- or Hac1-independent mechanisms contributing to the viability of the *ΔLD ire1Δ* cells and are possibly involved in the modulation of UPR^{LBS}. In addition, a genetic screen identified adaptive aneuploidy that conferred resistance to tunicamycin induced ER stress [228]. The chromosome duplication of chromosome II is sufficient to protect cells from ER stress, independent of the UPR [228].

It seems that Ire1 modifies its output (type of response) according to the input (type of stress). The removal of the luminal domain in *ΔLD ire1Δopi3Δ* cells reduces UPR^{LBS}, indicating the dispensability of the luminal domain in stress sensing due to changes in PC/PE ratio of the ER membrane. Data from our genetic screen, DTT treatment and inositol depletion experiment, however, indicate the importance of the luminal domain in stress sensing and UPR activation. It is therefore inconclusive how stress affects each domain of Ire1. It is, however, conclusive, that in the context of NAFLD, when there is a change in PC/PE ratio of the ER membrane, the activation of Ire1 is independent of its luminal domain, suggesting the possibility of other factors regulating Ire1 LD activity under different stresses. Recent emerging evidence points to factors that associate with the luminal domain of Ire1 modulating its activation and response to ER stress. The ER chaperone protein disulphide isomerase A6 (PDIA6), directly binds to cysteine 148 within the luminal domain of Ire1 α , reducing a disulphide bond thereby attenuating ER stress by preventing oligomerisation. The deletion of PDIA6 causes the hyper activation of Ire1 α as evident by increased *HAC1* splicing and the upregulation of downstream UPR targets [160]. Another ER chaperone, heat shock protein 47 (Hsp47), was identified as a regulator of Ire1 α signalling. Hsp47 functions by binding to Ire1 α luminal domain, resulting in the displacement of BiP and enhancing Ire1 α oligomerisation [161]. The association of such proteins to the luminal domain of Ire1 α leads us to speculate that although components of the ER modulate its activity to determine signalling amplitude and kinetics, the luminal domain itself may have an additional unknown regulatory role in the UPR.

4.3 The oligomerisation state of Ire1 under different types of stresses

One prominent observation from our *in vivo* Split Venus experiment revealed a difference in Ire1/ Δ LD clustering and *HAC1* splicing upon different stresses (proteotoxic stress *versus* inositol depletion and *opi3* Δ). It is tempting to speculate that the genes identified to be upregulated or downregulated in our genetic screen could act as factors affecting the activation of Ire1 or Δ LD (clustering, phosphorylation status) directly or indirectly since it was previously reported that signalling cascades may control Ire1 clustering [130]. We questioned the size of Ire1 clusters observed during UPR^{PT}. The oligomerisation status of Ire1 has been rather enigmatic. First evidences of Ire1 oligomerisation emerged with co-immunoprecipitation analysis of differentially epitope tagged Ire1 and density gradient fractionation of cell lysates that suggested that the oligomerization status of Ire1 is dimeric [31, 35, 38]. Indeed, in solution, recombinant Ire1 luminal domain and the CSSR (Subregion II-IV of the luminal domain; refer to Figure 7) exists as dimers [229, 230]. Crystal structures, however, argue that the CSSR forms higher order oligomers since one CSSR molecule is in contact with two CSSR molecules via two separate interfaces (IF1^L and IF2^L – “L” for luminal) [39]. IF1^L forms a symmetric dimer containing a deep groove while IF2^L allows further oligomerization with other Ire1 molecules [39]. The formation of higher order oligomers *in vivo* was later demonstrated by the cluster formation of Ire1 represented by discrete foci in the ER [42]. *In vivo* validation by single amino acid mutation in each CSSR interface demonstrated that interfaces IF1^L and IF2^L were required for LD oligomerization [39, 42]. *In vitro*, in addition to being co-immunoprecipitated with the unfolded protein substrate CPY (CPY*), purified Ire1 LD showed a shift from monomers, dimers and tetramers towards heterogenous large oligomers in the presence of another model unfolded substrate Δ EspP [24]. Furthermore, a model for higher order assembly of Ire1 was proposed, involving a two-dimensional arrangement of Ire1 and the incremental addition of symmetric back-to-back Ire1 dimers that consists of approximately 20-30 molecules of Ire1 [205]. This model is consistent with the size of Ire1 foci that was observed *in vivo* by fluorescence microscopy [49, 206]. The size of the Ire1 foci observed in our Split Venus experiment during UPR^{PT} suggests a similarity in the size of foci observed, suggesting a clustering of approximately 20-30 Ire1 molecules [205]. We specifically chose one of our strongest hits, *OPI3*, important for phospholipid homeostasis. The model that emerged from our data proposes that Ire1 clustering propensity depends on the type of stress imposed onto the cells. Studies of Ire1 oligomerisation during sustained ER stress by inositol depletion *versus* acute proteotoxic stress by DTT shows the difference in cluster formation between the different

stressors [64, 67, 202] (See Table 8 for a summary of differences in cluster formation between this study and published literature). As evident by fluorescent microscopy of HA tagged Ire1 protein, cells undergoing mild ER stress in the form of inositol depletion formed low intensity punctate structures in contrast to DTT treatment, suggesting that Ire1 self-associates to a homo-dimer to a less detectable extent without obvious clustering [67, 202]. Due to such low degree of clustering, there was an absence of observable Ire1 α clusters in HeLa cells expressing Split Venus tagged Ire1 α when membranes were saturated with palmitic acid, although puncta formation occurred during proteotoxic stress [203]. This was similar to the findings of this thesis. With our Split Venus method, while Ire1 cluster formation is evident during DTT treatment, there were no observable Ire1 clusters during inositol and PC depletion, although *HAC1* splicing occurred. The absence of puncta formation in Ire1 during inositol depletion and PC depletion hints at the ability of Ire1 distinguishing and co-ordinating its oligomeric state between acute, mild and chronic ER stress. Indeed, Ire1 was previously shown to distinguish its catalytic mechanisms for *XBP1/HAC1* splicing and RIDD activity, exhibiting a difference between oligomeric and dimeric states respectively [56]. Low intensity Ire1 clusters have previously been demonstrated when inositol was depleted from cells [64, 67]. We can surmise that the difference in methods used for Ire1 cluster detection led to the difference in observations due to a weaker and less detectable Venus fluorophore [231] (See Table 8 below for the summary of difference in Ire1 constructs and microscopy methods used). Unexpectedly, during inositol depletion, while it has been shown in ΔIII Ire1 that *HAC1* splicing occurs, we were unable to replicate that with our ΔLD construct.

What was interesting was that *HAC1* splicing is preserved even in the absence of higher order oligomers. For *HAC1* splicing to occur, it is essential for Ire1 kinase/RNase domain to be clustered to efficiently process *HAC1* mRNA [49, 205]. We therefore propose that while Ire1 form higher order oligomers consisting of 20-30 Ire1 molecules per puncta, Ire1 exist minimally as a non-clustered, self-associated dimer during lipid bilayer stress (inositol depletion and *opi3 Δ*) since *HAC1* splicing was preserved (Figure 31). This finding implies that chronic ER stress due to lipid bilayer stress results in an intracellular environment that enable dimerised Ire1 to exert its activity. While the molecular mechanism of dimerised Ire1 has not been demonstrated *in vivo*, there has been evidences of Ire1 and Ire1 LD existing in a dimeric state [31, 35, 38]. In addition, for Ire1 to exert its activity, it is possible that membrane properties are involved, affecting Ire1 structurally, allowing the activation of dimerised Ire1.

An additional factor that can be considered is the change in membrane phospholipid packing or curvature on Ire1 clustering. As a result of *OPI3* deletion, the intermediate mono-methylated PE (MMPE) accumulates in the ER bilayer. MMPE has physical properties similar to PE and the accumulation of MMPE will contribute to negative curvature stress similar to what has been reported for PE [232]. In addition, the replacement of PC with MMPE contributes to the stiffening of the membrane [115].

We were unable to determine if the absence of Ire1 cluster formation observed in the *opi3Δ* cells is a direct or indirect effect of PC depletion. In yeast, other than serving its role as a direct peptide binding groove via IF1^L and a site for oligomerisation via IF2^L, the LD associates to other regulatory/adaptor molecules such as Kar2 and OS9 [158]. Other unknown modulators of Ire1 (known modulators for Ire1α was previously mentioned in Section 4.2) may be up- or down-regulated due to lipid bilayer stress, possibly affecting Ire1 clustering propensity, explaining the lack of Ire1 clusters in *ire1Δopi3Δ* cells expressing Ire1. The lack of oligomerisation observed in Ire1 expressing *ire1Δopi3Δ* cells was restored once exposed to acute protein stress, suggesting the differences in adaptive response between UPR^{PT} and UPR^{LBS} on Ire1 clustering propensity. As future work, to overcome the limitation of weak Venus detection, a more photostable and brighter fluorophore, for example mNeonGreen, should be used to detect Ire1 cluster formation [231]. This will perhaps reveal if there is dimer forming propensity of Ire1 molecules during lipid bilayer stress or if they do indeed remain as inactive monomers. The activity of Ire1 is dependent on its autophosphorylation for the activation of the ribonuclease domain for *HAC1* mRNA splicing [45]. Using a Phos-tag gel, the differences in phosphorylation status of Ire1 and Ire1ΔLD during UPR^{PT} and UPR^{LBS} should also be characterised to distinguish phosphorylation states during different stresses. Regardless, the difference in foci formation between Ire1 molecules exposed to chronic protein stress and lipid bilayer stress is rather distinct.

Table 8: Comparison of lipid bilayer stress sensing in this study to ΔIII Ire1 [64] and amphipathic helix sensing of Ire1 [67].

	SOURCE			Similarities/Differences
	This study	Promlek <i>et al.</i> [64]	Halbleib <i>et al.</i> [67]	
Types of Ire1/Ire1 variant used by fluorescence microscopy	Ire1-VN/VC Δ LD-VN/VC	Ire1-HA bZIP-Ire1-HA	Ire1 3xHA-GFP	GFP is a stronger fluorophore compared to Venus [231], possibly explaining the lack of observable Ire1 cluster formation during inositol depletion in our study.
Method used to study cluster formation of Ire1/Ire1ΔLD or ΔIII Ire1	Live cell confocal imaging of Ire1-VN/VC and Δ LD-VN/VC	Indirect immunofluorescence of Ire1-HA Stained by mouse anti-HA antibody followed by FITC-labeled secondary antibody	Live cell confocal imaging of Ire1 3xHA-GFP	Live cell imaging was performed in this study, like in Halbleib <i>et al.</i> The laser power published in Halbleib <i>et al.</i> was different between DTT (2%) and inositol depletion (10%), suggesting the saturation of signal and high background observed in their images [67]. In our confocal imaging, we used a consistent laser power of 20% (514 nm) across all our samples and conditions for fair comparison.

Table 8: Comparison of lipid bilayer stress sensing in this study to ΔIII Ire1 [64] and amphipathic helix sensing of Ire1 [67].

	SOURCE			Similarities/Differences
	This study	Promlek <i>et al.</i> [64]	Halbleib <i>et al.</i> [67]	
Experimental conditions for DTT treatment (UPR^{PT}) and microscopy observation	<p><u>10 mM DTT, 1 hour</u></p> <p>Ire1-Venus cluster formation was observed. (Figure 28)</p> <p>No cluster formation for ΔLD-VC/VN (Figure 28)</p>	<p><u>10 mM DTT, 30 min</u></p> <p>Ire1 cluster formation was observed. (Figure 6B)</p> <p>No microscopic images of ΔIII Ire1.</p>	<p><u>10 mM DTT, 1.5 hours</u></p> <p>Ire1-GFP cluster formation was observed. (Figure 2A)</p> <p>No microscopic images of ΔIII Ire1.</p>	<p>DTT treatment led to Ire1 cluster formation across all three studies.</p> <p>Whereas our data did not detect ΔLD-Venus clusters during DTT treatment, the other studies did not perform microscopy using the ΔIII Ire1.</p>
<i>HAC1</i> splicing upon DTT treatment (UPR^{PT})	<p><u>10 mM DTT, 1 hour</u></p> <p><i>HAC1^s</i> was observed for Ire1 and ΔLD using the Split Venus strains. (Figure 31)</p>	<p><u>10 mM DTT, 30 min</u></p> <p><i>HAC1^s</i> was observed in Ire1 and ΔIII Ire1. (Figure 5D)</p>	<p><u>4 mM, 1.5 hours</u></p> <p><i>HAC1^s</i> was observed in Ire1 cells and ΔIII Ire1 (Figure 4A)</p>	<p><i>HAC1</i> splicing was demonstrated in all three studies in both Ire1 and ΔLD or ΔIII Ire1.</p>

Table 8: Comparison of lipid bilayer stress sensing in this study to ΔIII Ire1 [64] and amphipathic helix sensing of Ire1 [67].

	SOURCE			Similarities/Differences
	This study	Promlek <i>et al.</i> [64]	Halbleib <i>et al.</i> [67]	
Experimental conditions for UPR^{LBS} (inositol depletion) and PC depletion) and microscopy observation	<u>4 hours inositol depletion</u> No cluster formation observed for Ire1-VC/VN and ΔLD -VC/VN (Figure 29)	<u>4 hours inositol depletion</u> Cluster formation observed for Ire1. (Figure 6B) No microscopic images of ΔIII Ire1.	<u>3 hours inositol depletion</u> Ire1-GFP cluster formation was observed. (Figure 2A) No microscopic images of ΔIII Ire1.	We did not observe Ire1 clusters upon inositol depletion contrary to the low intensity clusters reported by Promlek <i>et al.</i> and Halbleib <i>et al.</i> As mentioned above, it may be that we did not over-saturate our laser power to detect these low intensity clusters [64, 67]. The second reason may be due to the lower intensity of the Venus fluorophore compared to GFP [231]. We are correcting the strains in our manuscript to mNeonGreen.
	<u>OPI3 deletion</u> No cluster formation observed for Ire1-VC/VN and ΔLD -VC/VN <i>ire1Δopi3Δ</i> cells. (Figure 28)	<u>bZIP-Ire1-HA</u> Cluster formation was observed in non-stress and inositol depletion.		

Table 8: Comparison of lipid bilayer stress sensing in this study to Δ III Ire1 [64] and amphipathic helix sensing of Ire1 [67].

	SOURCE			Similarities/Differences
	This study	Promlek <i>et al.</i> [64]	Halbleib <i>et al.</i> [67]	
HAC1 splicing upon inositol and PC depletion (UPR^{LBS})	<p><u>4 hours inositol depletion</u></p> <p><i>HAC1^s</i> was observed for Ire1-VN/VC but not ΔLD-VN/VC expressing <i>ire1Δ</i> cells.</p> <p>(Figure 31)</p>	<p><u>5 hours inositol depletion</u></p> <p><i>HAC1^s</i> was observed in Ire1 and ΔIII Ire1 expressing cells.</p> <p>(Figure 5C)</p>	<p><u>3 hours inositol depletion</u></p> <p><i>HAC1^s</i> was observed in Ire1 and ΔIII Ire1 expressing cells (Figure 4A).</p>	<p>While there was no observable Ire1 Venus cluster formation with confocal microscopy, our RT-PCR experiment of <i>HAC1^s</i> demonstrates Ire1 activation during inositol depletion. While this was similarly observed in the ΔIII construct, there was no <i>HAC1</i> splicing observed in ΔLD-VN/VC expressing <i>ire1Δ</i> cells.</p> <p><i>HAC1</i> splicing was however observed in both <i>IRE1</i> and ΔLD <i>ire1Δopi3Δ</i> cells.</p> <p><i>HAC1</i> splicing in ΔLD <i>ire1Δopi3Δ</i> cells and bZIP-Ire1 cells indicate the luminal domain independent sensing during UPR^{LBS}.</p>
	<p><u>OPI3 deletion</u></p> <p><i>HAC1^s</i> was observed for Ire1-VN/VC and ΔLD-VN/VC expressing <i>ire1Δopi3Δ</i> cells.</p> <p>(Figure 31)</p>	<p><u>bZIP-Ire1</u></p> <p><i>HAC1^s</i> was observed in bZIP-Ire1 expressing cells.</p>		
Ire1 LBS sensing variant	<p>ΔLD and Ire1 (R537Q)</p> <p>(Figure 8 and 32 respectively)</p>	<p>ΔIII Ire1</p> <p>(Figure 1)</p>	<p>F531R and V535R</p> <p>(Figure 1)</p>	<p>Region III of Ire1, Ire1 (F531) and Ire1 (V535) are involved in UPR^{PT} and UPR^{LBS} sensing. The constructs used in our study sense only UPR^{LBS}.</p>

4.4 Modulation of UPR^{LBS} by an arginine residue within the transmembrane domain of Ire1

The importance of the transmembrane domain in UPR signal propagation have been previously demonstrated [66]. Membrane tethering is crucial for propagation of the UPR signal [66]. Sequence mutation by Crispr-Cas9 confirmed the importance of transmembrane residues in the robust sensing of membrane saturation by the α -helix of Ire1 α . In contrast to a previous study where the transmembrane helix of Ire1 was completely replaced, it was recently found that specialised conserved features within the transmembrane helix are important to respond to lipid bilayer stress [82]. Indeed, it was found that within the transmembrane domain of Ire1 α , two residues W457 and S450 are required for stable Ire1 dimer formation during membrane saturation by palmitic acid. The increase in lipid packing of the membrane results in the transition of the aromatic ring of the tryptophan (W) residue orientating to face each other within the dimerisation interface to enable Trp457-Trp457 interactions. This association is further supported by the serine (S) residue that forms hydrogen bonds for the stability of the Trp457-Trp457 interaction during the Ire1 α transmembrane domain dimerization [83].

We presented here that the arginine residue at position 537 of the predicted amphipathic helix, within the transmembrane region of Ire1, uncouples UPR^{LBS} from UPR^{PT}. The disruption of the local charge, by the mutation of arginine 537 to glutamine, eliminated UPR activity. This suggests its crucial role in bringing about Ire1 monomers for association into stabilising conformations for higher order oligomer formation. When PC is depleted from the membrane, remodelling of the membrane occurs to maintain optimal intrinsic membrane curvature [114, 116]. Global changes to lipid acyl chain composition occurs, specifically PE levels increase to make up for the loss of PC, including the shortening and increased saturation of the PE acyl chain to decrease the non-bilayer forming propensity of PE [116]. As a result, we speculate that with decreased membrane fluidity and increased molecular lipid packing due to the increase in PE, two transmembrane domains of Ire1 are brought together, further driven by the electrostatic interaction between the positively charged arginine residue and the negatively charged glutamate residue (Figure 46). The mutation of arginine to the non-charged residue glutamine eliminates this driving force (Figure 46). This suggest two potential drivers of Ire1 activation 1) the luminal domain as a driver of Ire1 oligomerisation during proteotoxic stress 2) the electrostatic interaction between opposite transmembrane domains during LBS. Taken together with our oligomerisation data, we speculate that a dimeric form of Ire1 exists, driven by its transmembrane

domain, during UPR^{LBS} that is stabilised by its membrane environment, due to increased lipid packing (Figure 46). This contrasts with higher order oligomer formation driven by the luminal domain of Ire1 during proteotoxic stress (Figure 46). These two triggers of activation can work synergistically or independently as shown by our *IRE1* or $\Delta LD\ ire1\Delta opi3\Delta$ cells.

Given the importance of clustering in Ire1 activity, it can be assumed that in addition to acyl chain shortening and saturation mentioned above, other membrane properties are affected in response to altered membrane lipid composition. Protein-protein and protein-lipid interactions that are important for maintaining proper membrane structure and dynamics are affected during LBS. This change in interaction may stabilise or destabilise proteins or protein complexes. Based on the increase in UPR^{LBS} activity of Ire1, there is an indication that Ire1 dimerisation interface may be further stabilised during LBS thereby transducing a greater signal. The binding of specific lipids to the juxtamembrane region and hydrophobic α -helix region of type 1 transmembrane proteins is crucial for protein stability and activity [233]. In certain cases, lipid binding enhances oligomerisation and serves as a mechanism to regulate or control protein activity. How lipid binding regulates protein activity, in this case, Ire1 activity is still an unanswered question.

Alternatively, although there is no experimental evidence in this thesis to back this up, based on the hypothesis put forth by Halbleib *et al.* [67], during lipid bilayer stress, the mutation of R537Q may similarly disrupt the insertion of the residue into the lipid bilayer, resulting in less membrane compression and thus a lack of Ire1 oligomerisation and *HAC1* splicing. This hypothesis may be flawed since R537 only responds to UPR^{LBS} while the F531 and V535 residues respond to both UPR^{PT} and UPR^{LBS} [67]. This could be due to the position of the arginine residue, laying at the interface between the amphipathic helix and transmembrane domain of the entire Ire1 transmembrane region and the disruption of local charge. To further test this hypothesis, our future work should also include mutation of the negatively charged residue glutamine to determine if like R537Q, whether the loss of charge of this residue uncouples UPR^{PT} and UPR^{LBS}.

Additionally, the introduction of the R537Q mutation hints at the complexity in *HAC1* mRNA transcriptional regulation and suggest Hac1 independent transcription programme to cope with LBS. To date, although Ire1-dependent *HAC1* mRNA splicing is the most commonly looked at on/off switch as an output of Ire1/UPR activity, alternative modes of *HAC1* mRNA splicing regulation exists. One group

demonstrates the autoregulation of *HAC1* during period of extreme and prolonged ER stress by a positive feedback loop of Hac1 binding to its promoter [208]. There is a delicate balance faced by Ire1 in responding to the stress it encounters and a finely tuned response for the activation of particular genes to adapt to cellular changes [209]. The distinct pathway termed “Super-UPR”, that is activated in part, by *ire1* Δ cells that do not splice *HAC1* shows the distinction of this pathway and may explain the lack of *HAC1*^S observed in the *IRE1(R537Q)* and *I Δ LD(R537Q) ire1 Δ opi3 Δ* cells [209]. This also suggest an undiscovered alternative Hac1-independent pathway of homeostatic adaptation to UPR activation. Although this study lacks the molecular details of the Hac1-independent pathway observed, the evidences provided above support the existence of multiple mechanism of *HAC1* regulation that allows for the integration of multiple cellular stimuli to mount an appropriate response. Other biological processes that were identified by our genetic screen suggest the importance of other pathways such as protein glycosylation and mannosylation, vesicular trafficking and ERAD that compensates UPR^{LBS}. A question that remains yet unanswered is the specificity of the R537Q mutation during lipotoxic stress. Is the R537Q mutation specific to only PC/PE shifts or does it respond to membrane saturation or desaturation? Our future work on the R537Q mutant should involve other forms of lipid perturbation such as palmitate loading and inositol depletion. Palmitate supplementation increases membrane saturation and has been shown to directly activate Ire1 α Δ LD [83].

Figure 46: Model of Ire1 activation during UPR^{PT} and UPR^{LBS} . Ire1 is capable of distinguishing and integrating two different stimuli for oligomerisation. The first is luminal domain dependent where Ire1 forms higher order oligomers. The second is transmembrane domain dependent where Ire1 forms dimers. This transmembrane domain dimerization is driven by the electrostatic attraction between two oppositely charged residues (arginine and glutamate) in the α -helix. Compromising mutations by R537Q eliminates *HAC1* mRNA splicing suggesting Hac1 independent adaptation mechanisms. Downstream signal transduction differs between both stress stimuli confirming a divergent transcriptional response.

4.5 A Hac1-specific subset of genes is upregulated during lipid bilayer stress

Initially, cDNA microarray analysis reported approximately 400 UPR target genes upregulated upon ER stress [50]. Subsequently, further novel Hac1 gene targets were identified by the expression of a constitutively active Hac1 [185]. UPR target genes have been properly characterized, however, in *ire1Δ* cells, it has only been shown that homeostasis is achieved through remodelling of the proteome of the cell, instead of the lipidome [65]. To better understand the consequence of this cellular remodelling, our DNA microarray data revealed target genes that are upregulated specifically to LBS. ChIP data confirms that 5 genes out of 6 (*PUT1*, *PIR3*, *ACA1*, *GAC1* and *GPH1*) were indeed not upregulated by Hac1 binding during UPR^{PT}. The genes *PBI2*, *PIR3* and *PUT1* were found to be novel Hac1 binding genes in response to UPR^{LBS}. Like the stronger UPR induction observed with our β-galactosidase assay of UPR^{LBS}, a higher magnitude of UPR activation termed as “Super-UPR” has been previously described [209]. “Super UPR” is defined as higher levels of *HAC1* mRNA due to an accumulation of signals from both Ire1-dependent *HAC1* mRNA splicing from misfolded proteins and an Ire1-independent regulation of *HAC1* mRNA control from inositol depletion/temperature shift [209]. Similar to our findings, the authors identified an alternative transcriptional programme activated that is slightly altered from the normal UPR^{PT}, for the cells to adapt and survive.

Processes such as amino acid and carbohydrate metabolism are typically upregulated during ER stress for the purpose of replenishing internal depleted stores, validating the association of Hac1 to *PUT1* [234]. The integrity of the cell wall and the UPR are intimately linked, with Ire1 mutants displaying cell wall defects, suggesting the implication of maintaining cell wall integrity during LBS with *PIR3* upregulation [235, 236]. *PBI2* was identified to be significantly upregulated during tunicamycin treated ER stress by ChIP-qPCR. *PBI2* is upregulated during DNA replicative stress and plays a role in vacuole fusion [237, 238]. It is therefore not surprising that *PBI2* is upregulated given that DNA replicative stress genes are typically upregulated during cellular stress. *PBI2* contains a UPRE motif in its promoter and was identified as a gene upregulated by ER stress when Hac1 was constitutively active. *PBI2* has also been found to be upregulated during inositol supplementation, further linking it to the Ire1 pathway, but not differentially regulated with the over-supplementation of choline. On the contrary, the deprivation of both these molecules are expected to trigger lipid bilayer stress induced UPR which leads to the upregulation of *PBI2* suggesting its differential response to different lipid precursors. The potential of

PBI2 to respond differently to triggers of ER stress further emphasises the differential response orchestrated by the UPR and reinforcing a previous similar conclusion from the lab [132]. The functional relevance of each of these gene to lipid bilayer stress has not been studied, of which can be further characterized by mutational studies. Differential targets of Ire1 can be identified with deep sequencing of the targets of Hac1 following ChIP for a better understanding of the expanse of gene regulation by Hac1 during UPR^{LBS}.

Multiple levels of regulation exist at the transcriptional level post *HAC1^s* translation. Gcn4, a known transcriptional regulator of amino acid availability, was found to work synergistically with Hac1 at the promoter of UPR targets [162]. This suggest the role of additional transcriptional factors for differential gene output/transcription for signal propagation in respond to different stresses. In line with our microarray data, it is not surprising that 3 out of the 6 upregulated genes were not Hac1 binding targets. Although the *UPRE*, which is present in all UPR targets, is thought to contain a Hac1 binding site, an analysis of 381 promoter sequences of UPR target genes failed to recognise an identifiable *UPRE* in most of them, further contributing to evidence of Hac1-independent regulation of the UPR involving *ACA1*, *GAC1* and *GPH1*. A possible explanation to this paradox is the presence of other yet identified novel UPR motifs in addition to the three (*UPRE-1*, *UPRE-2* and *UPRE-3*) already known. The diversity of these undiscovered *UPRE* motifs could contribute to post transcriptional regulation of Hac1 and the recruitment of other *trans*-acting factors, whether alone or in concert with Hac1 for eliciting a more complex transcriptional programme [162]. It would be interesting to identify other regulatory factors recruited to *UPRE* sites in conjunction with Hac1 to mount a divergent transcriptional programme for adaption to different stresses. To answer this question, in our future work, we could adapt the genetic screen used in the beginning of the study and replace the *CYC1-UPRE* promoter to promoters of *PBI2*, *PIR3* or *PUT1* to determine which gene deletion causes the activation of the UPR.

Conclusion

As discussed above there is now clear evidence for a link between the UPR and lipid homeostasis; in years to come, we anticipate a wave of new findings that will further reinforce this view. Unlike the classic roles of the UPR in maintaining protein homeostasis, its regulatory role in lipid metabolism appears to be more complex. Below, we attempt to integrate our findings and consolidate this rather intricate mechanism. First, the UPR sensor Ire1 directly senses lipid bilayer stress (saturation and PC depletion) through the amphipathic helix of its transmembrane domain, an activity that is independent of its luminal domain. Second, the key factors in the UPR might have evolved additional features to cope with lipid bilayer stress. It seems that the UPR, once activated, responds to lipid bilayer stress in a manner that is similar to its classic function. This includes transcriptional activation of lipid synthesis genes and other stress responsive factors (e.g. chaperones), controlling mRNAs of genes involved in lipogenesis and lipoprotein metabolism and tuning overall translation through its downstream targets. Hac1 however, additionally binds to an alternative subset of genes to buffer homeostatic changes during UPR^{LBS}. Third, our current understanding regarding the mechanistic roles of the UPR in lipid disequilibrium is far from being fully understood. Nevertheless, with our current knowledge, it might already be feasible to design pharmacological interventions that modulate UPR activity under lipid bilayer stress. For example, in patients with obesity, non-alcoholic steatohepatitis, or type II diabetes, chronic ER stress occurs [239, 240]. Drugs have been developed that circumvent NASH and improves glucose tolerance by preventing ceramide accumulation that are inhibitors of acid sphingomyelinase called FIASMA or inhibitors of SPT named myriocin or FTY-720; a marketed for the treatment of multiple sclerosis [241, 242]. Alternatively, direct targeting of ceramide for degradation by the enzyme ceramidase, which catalyses the degradation of ceramide to sphingosine, has been shown to be effective in reducing high fat induced NASH and insulin resistance [243]. The potential of newly developed drug targets should be able to circumvent inflammation that results from the hyperactivation of the UPR during conditions of altered lipid species due to either a high fat diet or a dysregulation of lipid metabolism and/or synthesis. With the ongoing research and explosion of technology, we are optimistic that the UPR will become an excellent target for drug therapy and metabolic diseases, which thus eventually will be controllable, if not curable.

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Appendix

Table S1. The UPR is upregulated in 448 gene deletions in *IRE1* cells only.

GENE	<i>IRE1</i> *	<i>IRE1</i> Δ <i>LD</i>	GENE	<i>IRE1</i> *	<i>IRE1</i> Δ <i>LD</i>	GENE	<i>IRE1</i> *	<i>IRE1</i> Δ <i>LD</i>
<i>YPL073C</i>	1.56	1.49	<i>MAL11</i>	1.82	1.35	<i>CYC7</i>	1.65	1.25
<i>YMR053C</i>	2.93	1.49	<i>CAX4</i>	2.39	1.35	<i>VAC14</i>	2.38	1.25
<i>ATG38</i>	1.70	1.49	<i>SCS7</i>	2.19	1.34	<i>NAP1</i>	1.54	1.25
<i>CCS1</i>	1.77	1.49	<i>CAF20</i>	2.00	1.34	<i>UTR4</i>	1.67	1.25
<i>RPN4</i>	1.52	1.47	<i>ARF3</i>	1.56	1.34	<i>ARD1</i>	1.90	1.25
<i>AQY2</i>	1.84	1.47	<i>YKL133C</i>	1.53	1.34	<i>ANY1</i>	2.03	1.24
<i>SAM3</i>	1.97	1.46	<i>UFO1</i>	1.92	1.34	<i>PAN3</i>	1.87	1.24
<i>CCW14</i>	2.15	1.46	<i>SWC7</i>	2.05	1.34	<i>RTT103</i>	1.81	1.24
<i>SIP4</i>	1.80	1.46	<i>YER014W</i>	2.10	1.33	<i>GDS1</i>	1.55	1.23
<i>DCI1</i>	2.14	1.46	<i>PFD1</i>	2.23	1.33	<i>WSC2</i>	2.20	1.23
<i>YMR052W</i>	2.23	1.46	<i>MSM1</i>	1.66	1.32	<i>TPK3</i>	1.97	1.22
<i>PIG1</i>	1.58	1.45	<i>YGR168C</i>	1.60	1.32	<i>YPL034W</i>	2.21	1.22
<i>VAM3</i>	1.54	1.45	<i>RDL2</i>	1.89	1.32	<i>EAF3</i>	2.12	1.22
<i>NPP2</i>	1.87	1.45	<i>HAA1</i>	1.83	1.32	<i>YIR044C</i>	1.84	1.22
<i>HKR1</i>	1.51	1.45	<i>RGA2</i>	2.22	1.32	<i>MED1</i>	1.74	1.22
<i>SUL1</i>	1.79	1.44	<i>SBE2</i>	1.83	1.31	<i>VPS71</i>	1.60	1.22
<i>AIM41</i>	1.84	1.44	<i>ALG6</i>	3.40	1.31	<i>TED1</i>	1.73	1.21
<i>YBL083C</i>	2.55	1.44	<i>SHE4</i>	2.03	1.31	<i>IRC22</i>	1.77	1.21
<i>YOR283W</i>	1.94	1.43	<i>RPN13</i>	1.75	1.31	<i>SPO11</i>	2.81	1.21
<i>HSP150</i>	1.53	1.43	<i>YGL218W</i>	2.06	1.30	<i>BUL1</i>	1.86	1.21
<i>ALG3</i>	4.40	1.42	<i>YJL075C</i>	1.55	1.30	<i>CCW12</i>	1.77	1.21
<i>BDF2</i>	1.60	1.42	<i>HOP2</i>	1.67	1.30	<i>VIP1</i>	2.36	1.20
<i>LYS2</i>	2.78	1.42	<i>PKR1</i>	1.60	1.30	<i>VMA4</i>	2.19	1.20
<i>UBC8</i>	2.44	1.40	<i>SPO23</i>	1.77	1.29	<i>MHP1</i>	2.32	1.20
<i>FCY1</i>	2.16	1.40	<i>YDR134C</i>	1.52	1.28	<i>LPX1</i>	3.15	1.19
<i>YLR235C</i>	1.73	1.39	<i>TGL5</i>	1.73	1.28	<i>ADE17</i>	1.94	1.19
<i>PHR1</i>	1.99	1.39	<i>RTS1</i>	1.61	1.28	<i>YEL008W</i>	1.67	1.19
<i>TOP3</i>	1.70	1.38	<i>RPL34a</i>	1.61	1.28	<i>YJL028W</i>	2.31	1.19
<i>ECM38</i>	1.92	1.38	<i>GRS1</i>	1.90	1.28	<i>TRS85</i>	1.85	1.18
<i>CBP4</i>	1.75	1.38	<i>YML095C-A</i>	1.69	1.28	<i>SSP1</i>	1.52	1.18
<i>MGA2</i>	1.61	1.38	<i>EAF1</i>	1.85	1.27	<i>YGR011W</i>	1.87	1.18
<i>MXR2</i>	2.23	1.37	<i>LSB6</i>	1.64	1.27	<i>YPT7</i>	1.71	1.18
<i>JID1</i>	1.55	1.37	<i>CBF1</i>	1.73	1.27	<i>INP1</i>	1.71	1.17
<i>YLR365W</i>	1.54	1.36	<i>SST2</i>	1.58	1.27	<i>OPI1</i>	1.71	1.17
<i>YLR338W</i>	1.61	1.36	<i>CHS7</i>	1.59	1.26	<i>SAM1</i>	1.99	1.17
<i>YBL081W</i>	1.54	1.36	<i>LIP2</i>	1.90	1.26	<i>PEX12</i>	1.66	1.17
<i>DIA3</i>	2.37	1.36	<i>MAL32</i>	1.71	1.25	<i>RPS7b</i>	1.75	1.16
<i>YNL285W</i>	1.69	1.35	<i>EUG1</i>	2.35	1.25	<i>RAD55</i>	1.63	1.16

*The values represent the log₂ (fold change) ≥ 1.5 of UPR activation in *IRE1* cells only, upon deletion of the respective gene (Figure 12, blue). Values are calculated by the ratio of normalised GFP/mCherry signal.

Table S1. The UPR is upregulated in 448 gene deletions in *IRE1* cells only.

GENE	<i>IRE1</i> *	<i>IRE1</i> Δ LD	GENE	<i>IRE1</i> *	<i>IRE1</i> Δ LD	GENE	<i>IRE1</i> *	<i>IRE1</i> Δ LD
<i>MLH1</i>	2.00	1.16	<i>COS1</i>	1.69	1.06	<i>YML112W</i>	3.84	0.99
<i>ADH2</i>	1.82	1.16	<i>STB1</i>	1.61	1.06	<i>TOK1</i>	1.74	0.99
<i>ARG4</i>	2.15	1.16	<i>SWR1</i>	1.82	1.05	<i>HFA1</i>	2.75	0.99
<i>NBA1</i>	1.59	1.15	<i>YGL199C</i>	1.59	1.05	<i>YFL013W-A</i>	2.12	0.99
<i>YOL037C</i>	1.54	1.15	<i>SAP4</i>	1.56	1.05	<i>ERP1</i>	1.62	0.99
<i>ERG24</i>	1.60	1.15	<i>YBR116C</i>	1.52	1.05	<i>YLR236C</i>	3.35	0.99
<i>HER2</i>	1.81	1.14	<i>NBP2</i>	1.82	1.05	<i>RNH70</i>	1.51	0.98
<i>GRX4</i>	1.57	1.14	<i>AMF1</i>	1.63	1.05	<i>DCS1</i>	2.60	0.98
<i>CSN9</i>	1.72	1.13	<i>ERP4</i>	2.77	1.05	<i>OMA1</i>	1.63	0.98
<i>ARG81</i>	2.42	1.13	<i>CUE5</i>	1.58	1.04	<i>VPS64</i>	2.33	0.98
<i>STO1</i>	1.86	1.13	<i>YHR182W</i>	1.52	1.04	<i>YBR144C</i>	1.57	0.98
<i>IOC2</i>	1.96	1.13	<i>YPR064W</i>	2.11	1.04	<i>RPP1a</i>	3.18	0.97
<i>YFL010C</i>	1.93	1.12	<i>DJP1</i>	1.96	1.04	<i>PHO88</i>	2.96	0.97
<i>ATP15</i>	2.02	1.12	<i>GTR2</i>	1.62	1.04	<i>CBP2</i>	2.41	0.97
<i>BOR1</i>	2.15	1.12	<i>SPT10</i>	1.68	1.04	<i>ASE1</i>	2.40	0.96
<i>YPL071C</i>	1.59	1.12	<i>FCY2</i>	2.12	1.03	<i>FRE3</i>	1.73	0.95
<i>SCS3</i>	2.37	1.12	<i>PEP12</i>	1.69	1.03	<i>YGL114W</i>	2.41	0.95
<i>MSL1</i>	1.56	1.12	<i>YPL257W</i>	2.47	1.03	<i>ALG8</i>	2.15	0.94
<i>YHL037C</i>	3.35	1.11	<i>REC114</i>	1.90	1.03	<i>YEL010W</i>	1.77	0.94
<i>SEG2</i>	2.51	1.11	<i>YMR141C</i>	2.03	1.03	<i>ENT2</i>	1.50	0.94
<i>DIA4</i>	1.68	1.11	<i>SHM1</i>	1.70	1.03	<i>PUS6</i>	2.50	0.94
<i>INO2</i>	2.07	1.11	<i>RTC2</i>	1.85	1.03	<i>YJL118W</i>	1.69	0.93
<i>RTT101</i>	2.03	1.11	<i>YPR044C</i>	1.53	1.02	<i>STB5</i>	1.63	0.93
<i>RAD14</i>	1.91	1.10	<i>DMC1</i>	1.58	1.02	<i>VPS69</i>	1.85	0.92
<i>IRC18</i>	1.54	1.10	<i>ECM29</i>	2.48	1.02	<i>WHI5</i>	1.95	0.92
<i>VPS52</i>	2.13	1.10	<i>EST1</i>	2.20	1.02	<i>STP3</i>	2.00	0.92
<i>YEA6</i>	1.52	1.10	<i>YKL131W</i>	1.74	1.01	<i>YLR232W</i>	1.92	0.92
<i>THP1</i>	1.51	1.09	<i>IMP2</i>	1.91	1.01	<i>DUS3</i>	1.78	0.92
<i>YBP1</i>	1.65	1.09	<i>BST1</i>	1.53	1.01	<i>YMR252C</i>	3.10	0.92
<i>YBR219C</i>	1.59	1.09	<i>RIM15</i>	1.76	1.01	<i>SOP4</i>	2.72	0.92
<i>IZH1</i>	2.56	1.09	<i>ROX3</i>	1.52	1.01	<i>MRX14</i>	1.54	0.92
<i>ATF2</i>	1.74	1.08	<i>PFS1</i>	1.59	1.01	<i>SAP185</i>	1.95	0.92
<i>ATG41</i>	1.84	1.07	<i>SAS2</i>	1.56	1.01	<i>FMS1</i>	1.85	0.91
<i>RMI1</i>	3.14	1.07	<i>HLR1</i>	1.83	1.00	<i>TCB1</i>	1.81	0.91
<i>KEX1</i>	1.67	1.07	<i>MET17</i>	2.17	1.00	<i>BUD30</i>	1.76	0.91
<i>ATG39</i>	1.79	1.07	<i>MIG2</i>	1.56	1.00	<i>RPL13b</i>	1.67	0.91
<i>ERD1</i>	1.87	1.06	<i>VEL1</i>	1.56	1.00	<i>YMR057C</i>	1.56	0.90
<i>YPR027C</i>	2.57	1.06	<i>ATG7</i>	1.91	1.00	<i>ATC1</i>	1.98	0.90
<i>YDR514C</i>	2.05	1.06	<i>RCK1</i>	3.08	1.00	<i>PDR17</i>	3.79	0.90

*The values represent the log₂ (fold change) ≥ 1.5 of UPR activation in *IRE1* cells only, upon deletion of the respective gene (Figure 12, blue). Values are calculated by the ratio of normalised GFP/mCherry signal.

Table S1. The UPR is upregulated in 448 gene deletions in *IRE1* cells only.

GENE	<i>IRE1</i> *	<i>IRE1</i> Δ <i>LD</i>	GENE	<i>IRE1</i> *	<i>IRE1</i> Δ <i>LD</i>	GENE	<i>IRE1</i> *	<i>IRE1</i> Δ <i>LD</i>
<i>EXO1</i>	2.25	1.06	<i>GUT1</i>	2.15	1.00	<i>DRN1</i>	1.57	0.90
<i>PRP18</i>	1.69	1.06	<i>KRE2</i>	1.84	0.99	<i>TRX2</i>	2.82	0.89
<i>MTC1</i>	1.58	0.89	<i>GLO1</i>	2.07	0.75	<i>MDL2</i>	1.83	0.59
<i>FDO1</i>	1.89	0.89	<i>GAL83</i>	4.58	0.74	<i>MRPL44</i>	1.69	0.59
<i>POP2</i>	1.97	0.88	<i>PET117</i>	1.89	0.74	<i>YJR055W</i>	2.23	0.58
<i>YKU70</i>	1.66	0.88	<i>MIP6</i>	1.67	0.74	<i>ICS3</i>	2.15	0.58
<i>SRC1</i>	1.71	0.87	<i>YDL023C</i>	1.82	0.73	<i>GSF2</i>	1.71	0.58
<i>BNI1</i>	1.97	0.87	<i>CYB5</i>	1.59	0.73	<i>RIT1</i>	1.57	0.58
<i>HOM3</i>	2.82	0.87	<i>MUK1</i>	1.60	0.73	<i>RPL39</i>	2.42	0.58
<i>IST1</i>	2.93	0.87	<i>STE11</i>	2.01	0.72	<i>WSC4</i>	2.73	0.58
<i>OST3</i>	5.20	0.87	<i>HAP2</i>	1.66	0.72	<i>PEX14</i>	2.25	0.57
<i>PRM15</i>	1.58	0.87	<i>GIS1</i>	1.61	0.72	<i>ICS2</i>	1.67	0.57
<i>MNN11</i>	2.92	0.86	<i>YOR238W</i>	1.63	0.71	<i>MDM30</i>	1.72	0.57
<i>LAS21</i>	2.13	0.86	<i>BRO1</i>	1.76	0.70	<i>RAD16</i>	2.50	0.56
<i>TOS1</i>	1.71	0.86	<i>DTR1</i>	2.36	0.70	<i>YPR050C</i>	2.46	0.56
<i>PRR1</i>	1.61	0.85	<i>RRF1</i>	1.60	0.69	<i>RCE1</i>	1.84	0.55
<i>FSH3</i>	1.67	0.85	<i>YME1</i>	2.30	0.69	<i>PLB2</i>	2.23	0.55
<i>YHL026C</i>	2.09	0.85	<i>TPP1</i>	1.79	0.69	<i>LRS4</i>	1.55	0.55
<i>ADE5,7</i>	2.16	0.85	<i>TEX1</i>	2.22	0.69	<i>SAN1</i>	1.88	0.54
<i>MRPS35</i>	2.01	0.84	<i>YPL113C</i>	1.54	0.68	<i>PBY1</i>	1.77	0.54
<i>YOR055W</i>	1.83	0.84	<i>ABZ2</i>	2.31	0.68	<i>ATG26</i>	1.73	0.54
<i>YDL026W</i>	1.76	0.84	<i>CGR1</i>	2.35	0.68	<i>ATP7</i>	1.51	0.53
<i>YHI9</i>	2.35	0.83	<i>UBP9</i>	1.54	0.68	<i>PRC1</i>	1.64	0.53
<i>SLM2</i>	2.18	0.81	<i>RPL15b</i>	1.57	0.67	<i>TDA2</i>	1.67	0.53
<i>YVC1</i>	2.36	0.81	<i>CDA2</i>	1.61	0.67	<i>YPL050C</i>	2.39	0.53
<i>RPB4</i>	1.70	0.80	<i>VPS36</i>	2.67	0.66	<i>IZH2</i>	2.45	0.53
<i>YDR199W</i>	2.42	0.79	<i>ENO1</i>	1.57	0.65	<i>GRX8</i>	1.55	0.52
<i>YER182W</i>	2.08	0.79	<i>VPS34</i>	1.98	0.64	<i>RRT8</i>	1.76	0.52
<i>YNR033W</i>	1.67	0.79	<i>EOS1</i>	5.12	0.63	<i>RTF1</i>	2.00	0.51
<i>VPS65</i>	1.94	0.79	<i>SSP2</i>	1.55	0.63	<i>IPT1</i>	1.64	0.51
<i>FAB1</i>	1.65	0.78	<i>MRPL23</i>	2.17	0.63	<i>YNL109W</i>	2.18	0.50
<i>ESL2</i>	1.87	0.78	<i>POF1</i>	1.57	0.62	<i>APT2</i>	1.55	0.50
<i>GTT3</i>	1.67	0.78	<i>PER1</i>	1.90	0.62	<i>RPL6a</i>	1.55	0.49
<i>UBP15</i>	2.14	0.78	<i>NHX1</i>	1.55	0.62	<i>AMD1</i>	1.69	0.48
<i>YPR098C</i>	2.22	0.77	<i>MBB1</i>	1.58	0.61	<i>YOL046C</i>	1.69	0.48
<i>YNL338W</i>	1.72	0.76	<i>BUD21</i>	1.72	0.61	<i>GTO3</i>	1.70	0.47
<i>YOP1</i>	1.88	0.76	<i>DAS2</i>	1.90	0.61	<i>JLP2</i>	1.72	0.47
<i>ELO3</i>	1.64	0.76	<i>RNR4</i>	1.90	0.60	<i>PEX30</i>	1.53	0.47
<i>CLN2</i>	1.58	0.75	<i>MRPL4</i>	1.70	0.60	<i>YMR262W</i>	1.75	0.45

*The values represent the log₂ (fold change) ≥ 1.5 of UPR activation in *IRE1* cells only, upon deletion of the respective gene (Figure 12, blue). Values are calculated by the ratio of normalised GFP/mCherry signal.

Table S1. The UPR is upregulated in 448 gene deletions in *IRE1* cells only.

GENE	<i>IRE1</i> *	<i>IRE1</i> Δ <i>LD</i>	GENE	<i>IRE1</i> *	<i>IRE1</i> Δ <i>LD</i>	GENE	<i>IRE1</i> *	<i>IRE1</i> Δ <i>LD</i>
<i>ICL2</i>	1.71	0.75	<i>SWC3</i>	1.92	0.60	<i>YMR294W-A</i>	1.60	0.45
<i>KAP120</i>	1.51	0.75	<i>TMC1</i>	1.92	0.59	<i>MSC7</i>	1.52	0.42
<i>MVB12</i>	2.17	0.42	<i>YDR124W</i>	1.90	0.15	<i>SNG1</i>	2.02	-0.74
<i>FAT3</i>	2.34	0.40	<i>HES1</i>	1.63	0.13	<i>MIC12</i>	1.52	-0.77
<i>POX1</i>	1.53	0.40	<i>RPL37b</i>	1.54	0.11	<i>TIM21</i>	2.38	-0.86
<i>ALE1</i>	1.61	0.39	<i>YIL131C</i>	2.11	0.07	<i>OAC1</i>	1.56	-0.90
<i>VMA7</i>	1.66	0.39	<i>SPE2</i>	1.79	0.07	<i>IRC10</i>	1.69	-0.92
<i>LYS4</i>	1.97	0.38	<i>YKL177W</i>	1.97	0.06	<i>YAR047C</i>	3.62	-0.95
<i>YMR316C-A</i>	1.57	0.38	<i>APN1</i>	2.49	0.06	<i>TWF1</i>	1.96	-1.04
<i>MRPL15</i>	1.76	0.36	<i>GSH1</i>	1.72	0.00	<i>NGL1</i>	1.65	-1.04
<i>ZRT3</i>	1.52	0.35	<i>REC102</i>	1.58	0.00	<i>ERP5</i>	1.59	-1.05
<i>ECM3</i>	1.86	0.33	<i>TDH3</i>	1.56	0.00	<i>MRP13</i>	1.86	-1.05
<i>PLM2</i>	1.62	0.33	<i>GNT1</i>	1.86	-0.21	<i>HRT3</i>	1.92	-1.13
<i>PIC2</i>	1.58	0.32	<i>HXT2</i>	1.56	-0.22	<i>OGG1</i>	1.89	-1.14
<i>YOR268C</i>	1.56	0.32	<i>CIR1</i>	1.67	-0.23	<i>GAD1</i>	1.95	-1.15
<i>GMC1</i>	11.00	0.32	<i>JIP3</i>	2.30	-0.26	<i>GAL2</i>	1.99	-1.33
<i>ALD5</i>	1.67	0.32	<i>HGH1</i>	2.24	-0.28	<i>RIM101</i>	1.68	-1.47
<i>DAL81</i>	1.78	0.32	<i>SRL4</i>	1.60	-0.28	<i>COX17</i>	2.57	-1.47
<i>CBP3</i>	1.96	0.31	<i>CHA4</i>	1.51	-0.28	<i>SYH1</i>	1.57	-1.86
<i>OSW5</i>	1.53	0.31	<i>CAM1</i>	1.70	-0.31	<i>SEO1</i>	2.44	-2.10
<i>NAT5</i>	2.17	0.31	<i>TDA10</i>	1.59	-0.35	<i>YLR111W</i>	1.58	-3.12
<i>LAM5</i>	1.86	0.30	<i>YOR385W</i>	1.92	-0.37	<i>YAL065C</i>	2.35	-3.64
<i>YMR007W</i>	1.98	0.30	<i>UPF3</i>	1.64	-0.41	<i>SWH1</i>	1.61	-3.76
<i>SDH7</i>	2.01	0.28	<i>UTR2</i>	1.87	-0.45	<i>FRE6</i>	1.52	-3.88
<i>MDR1</i>	2.19	0.28	<i>YEL023C</i>	1.69	-0.45	<i>YAR043C</i>	1.62	-4.30
<i>ADI1</i>	1.53	0.24	<i>PDR10</i>	2.07	-0.56	<i>MMP1</i>	1.92	-4.66
<i>APE4</i>	1.60	0.20	<i>PAC10</i>	2.22	-0.57	<i>YLR108C</i>	1.67	-4.68
<i>VAC8</i>	2.00	0.19	<i>BOL3</i>	1.91	-0.57	<i>ATG10</i>	2.45	-6.60
<i>YGL109W</i>	1.83	0.18	<i>ASK10</i>	2.59	-0.66	<i>APC9</i>	1.64	-7.19
<i>GLC3</i>	4.60	0.16	<i>OLA1</i>	1.86	-0.68	<i>SPE1</i>	1.65	-21.55

*The values represent the log₂ (fold change) ≥ 1.5 of UPR activation in *IRE1* cells only, upon deletion of the respective gene (Figure 12, blue). Values are calculated by the ratio of normalised GFP/mCherry signal.

Table S2. The UPR is upregulated in 777 gene deletions in *IRE1 Δ LD* cells only.

GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *	GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *	GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *
<i>ATG3</i>	0.95	11.57	<i>UIP3</i>	-0.50	5.12	<i>NTG1</i>	1.00	3.34
<i>IRC19</i>	1.46	11.52	<i>ARP6</i>	1.10	5.10	<i>ISW2</i>	0.35	3.34
<i>YLR001C</i>	-2.91	9.72	<i>ENT4</i>	-0.53	5.03	<i>MRX6</i>	0.72	3.32
<i>XDJ1</i>	1.13	8.46	<i>ISU1</i>	0.86	4.93	<i>SVS1</i>	1.20	3.27
<i>IRC25</i>	0.03	8.14	<i>HOG1</i>	1.19	4.89	<i>WHI3</i>	0.88	3.24
<i>MSO1</i>	0.87	7.81	<i>MKK2</i>	0.06	4.83	<i>SNF7</i>	0.68	3.22
<i>YLL020C</i>	-0.55	7.74	<i>URK1</i>	0.90	4.60	<i>PET309</i>	0.23	3.22
<i>KNS1</i>	-2.09	7.69	<i>ATF1</i>	1.49	4.29	<i>VPS24</i>	0.74	3.21
<i>SDH2</i>	1.45	7.10	<i>KIN3</i>	1.00	4.28	<i>YLL054C</i>	0.55	3.21
<i>PSK1</i>	0.06	7.09	<i>SHG1</i>	1.06	4.25	<i>CUB1</i>	0.94	3.20
<i>MDM10</i>	-0.47	7.09	<i>MRPS16</i>	0.90	4.19	<i>CPR6</i>	1.17	3.20
<i>RAX2</i>	1.46	6.67	<i>CNE1</i>	1.24	4.18	<i>YER010C</i>	1.40	3.14
<i>EMP70</i>	0.38	6.53	<i>MAM3</i>	0.04	4.03	<i>KIN2</i>	1.43	3.12
<i>YLR149C</i>	0.76	6.06	<i>MRP17</i>	1.19	4.00	<i>BUD20</i>	1.05	3.12
<i>LAM6</i>	-0.51	6.03	<i>VTS1</i>	0.32	3.94	<i>XYL2</i>	0.17	3.12
<i>JIP4</i>	1.29	6.02	<i>SKG3</i>	0.87	3.91	<i>PEX29</i>	1.15	3.09
<i>SRL2</i>	1.31	5.70	<i>FUN19</i>	0.62	3.88	<i>ESC8</i>	0.06	3.08
<i>YLR042C</i>	0.21	5.69	<i>LHS1</i>	0.42	3.87	<i>IDH2</i>	0.00	3.08
<i>YLR118C</i>	1.30	5.63	<i>SIN4</i>	0.89	3.72	<i>GPM2</i>	0.51	3.05
<i>YLR053C</i>	-0.54	5.63	<i>PRM3</i>	1.49	3.64	<i>GCR2</i>	1.17	3.03
<i>LOT6</i>	-1.85	5.59	<i>BLI1</i>	-1.66	3.63	<i>YGR122W</i>	-0.74	3.02
<i>YPL197C</i>	0.05	5.56	<i>RDS2</i>	0.27	3.60	<i>YAR023C</i>	0.68	3.01
<i>NYV1</i>	0.73	5.51	<i>SPT8</i>	0.97	3.54	<i>KEX2</i>	0.88	3.00
<i>YLR112W</i>	0.33	5.51	<i>HSP82</i>	0.45	3.54	<i>BEM3</i>	1.31	3.00
<i>RFX1</i>	1.14	5.46	<i>ROT2</i>	1.47	3.47	<i>PHM8</i>	1.27	2.99
<i>YLL058W</i>	1.28	5.45	<i>YNL034W</i>	0.75	3.45	<i>MSN4</i>	1.00	2.98
<i>CMS1</i>	1.15	5.40	<i>PEX22</i>	0.94	3.44	<i>DPH1</i>	0.96	2.95
<i>YPL148C</i>	-0.15	5.39	<i>FTH1</i>	1.12	3.42	<i>PAC2</i>	1.47	2.93
<i>YBR206W</i>	1.40	5.38	<i>ELC1</i>	0.80	3.42	<i>ISC1</i>	1.02	2.92
<i>CSI2</i>	1.33	5.34	<i>SLX1</i>	0.95	3.38	<i>NRG2</i>	1.33	2.89
<i>YAL066W</i>	0.82	5.22	<i>YGR035C</i>	1.47	3.36	<i>RPS10b</i>	1.40	2.89
<i>BFR1</i>	0.47	5.21	<i>NCR1</i>	0.92	3.36	<i>YKL222C</i>	0.95	2.89
<i>KIP2</i>	0.55	5.18	<i>SIC1</i>	1.06	3.36	<i>MBA1</i>	0.73	2.88
<i>YOL019W</i>	-0.41	5.15	<i>DEF1</i>	1.00	3.36	<i>YDJ1</i>	0.66	2.87
<i>YOL085C</i>	1.15	2.86	<i>ETR1</i>	0.98	2.57	<i>ARC1</i>	0.31	2.40
<i>TDA9</i>	-0.12	2.86	<i>KRE28</i>	1.08	2.56	<i>PCS60</i>	0.67	2.40
<i>DIG2</i>	-0.37	2.84	<i>YPR003C</i>	1.43	2.56	<i>GCN5</i>	2.36	2.39
<i>PAU17</i>	-10.00	2.83	<i>NCA2</i>	0.36	2.55	<i>RPL26b</i>	1.46	2.39

*The values represent the log₂ (fold change) ≥ 1.5 of UPR activation in *IRE1 Δ LD* cells only, upon deletion of the respective gene (Figure 12, green). Values are calculated by the ratio of normalised GFP/mCherry signal.

Table S2. The UPR is upregulated in 777 gene deletions in *IRE1 Δ LD* cells only.

GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *	GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *	GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *
<i>CDC73</i>	1.30	2.81	<i>CAR1</i>	0.32	2.55	<i>YOR097C</i>	-0.40	2.39
<i>LEO1</i>	0.45	2.80	<i>YMR075C-A</i>	1.24	2.48	<i>RPS23a</i>	-1.19	2.29
<i>MFM1</i>	1.02	2.79	<i>ERP2</i>	-2.57	2.48	<i>RAX1</i>	0.85	2.29
<i>AAT2</i>	0.72	2.77	<i>YKL053W</i>	0.71	2.48	<i>ANB1</i>	0.66	2.29
<i>NFU1</i>	1.39	2.77	<i>YMR254C</i>	0.41	2.47	<i>PIP2</i>	0.00	2.28
<i>CWC21</i>	1.01	2.77	<i>YIL001W</i>	0.89	2.47	<i>ESC2</i>	1.12	2.26
<i>SCM4</i>	1.06	2.73	<i>USA1</i>	0.29	2.47	<i>RUB1</i>	-0.33	2.26
<i>SUB1</i>	1.32	2.73	<i>VBA1</i>	1.24	2.46	<i>YIL100W</i>	1.36	2.26
<i>CGI121</i>	1.29	2.71	<i>TEF4</i>	0.78	2.45	<i>PDB1</i>	0.80	2.26
<i>ESC1</i>	0.50	2.70	<i>YDR018C</i>	0.89	2.44	<i>BRE2</i>	-0.67	2.26
<i>PER33</i>	0.19	2.70	<i>YMR147W</i>	1.50	2.44	<i>YMR160W</i>	1.05	2.25
<i>PPE1</i>	0.74	2.68	<i>YGR017W</i>	0.11	2.43	<i>MIH1</i>	0.75	2.25
<i>RAD17</i>	0.06	2.68	<i>MET12</i>	1.00	2.43	<i>VAN1</i>	1.47	2.25
<i>SNX3</i>	-0.37	2.68	<i>NQM1</i>	0.68	2.43	<i>PEX6</i>	0.20	2.24
<i>SWS2</i>	0.41	2.67	<i>LDH1</i>	1.25	2.43	<i>CPT1</i>	1.07	2.24
<i>LOA1</i>	1.23	2.66	<i>TCM62</i>	1.36	2.42	<i>YIL056W</i>	0.70	2.24
<i>YIL092W</i>	1.07	2.66	<i>INM2</i>	0.90	2.41	<i>AIM33</i>	1.45	2.23
<i>UBP2</i>	0.82	2.64	<i>HOM2</i>	0.17	2.41	<i>YEL068C</i>	0.63	2.22
<i>YBL062W</i>	1.02	2.62	<i>SIF2</i>	1.20	2.41	<i>DID4</i>	0.73	2.22
<i>PCH2</i>	1.24	2.62	<i>PUS5</i>	0.22	2.41	<i>TMA64</i>	0.99	2.22
<i>YOR082C</i>	0.95	2.61	<i>URM1</i>	0.91	2.39	<i>RAS2</i>	0.87	2.21
<i>ALD4</i>	0.98	2.61	<i>ARG7</i>	0.66	2.38	<i>NMA111</i>	0.93	2.21
<i>SPP1</i>	0.58	2.60	<i>GAS3</i>	1.30	2.37	<i>GRX6</i>	0.94	2.20
<i>DAL4</i>	1.16	2.60	<i>KRE6</i>	1.15	2.36	<i>YPK3</i>	0.83	2.20
<i>MLH3</i>	1.39	2.60	<i>SPS2</i>	0.00	2.35	<i>HTZ1</i>	1.44	2.20
<i>May-24</i>	-0.56	2.59	<i>GAL4</i>	0.75	2.34	<i>GPX2</i>	0.95	2.19
<i>YNR042W</i>	1.09	2.59	<i>ATP11</i>	0.29	2.34	<i>APE1</i>	0.80	2.19
<i>YNL205C</i>	2.15	2.58	<i>YOL035C</i>	0.45	2.34	<i>IMD4</i>	0.99	2.18
<i>YOR314W</i>	1.11	2.57	<i>FMP49</i>	1.48	2.34	<i>CYB2</i>	0.33	2.18
<i>MUM2</i>	0.97	2.55	<i>ARC18</i>	1.26	2.34	<i>AIM36</i>	1.42	2.18
<i>ICE2</i>	1.34	2.54	<i>IXR1</i>	1.37	2.33	<i>YNL184C</i>	1.11	2.18
<i>AEP3</i>	1.20	2.54	<i>CIN5</i>	-0.48	2.32	<i>RCY1</i>	0.96	2.18
<i>KTR6</i>	0.63	2.53	<i>GAS2</i>	0.82	2.32	<i>MRX7</i>	0.85	2.18
<i>BNI4</i>	1.12	2.53	<i>FAR11</i>	1.26	2.31	<i>THP2</i>	1.27	2.17
<i>YBR063C</i>	0.93	2.52	<i>TEP1</i>	1.11	2.31	<i>ELA1</i>	1.00	2.17
<i>YDR209C</i>	-0.50	2.50	<i>DFG5</i>	0.71	2.31	<i>HOS2</i>	0.60	2.17
<i>GND1</i>	1.43	2.50	<i>TRS65</i>	1.46	2.31	<i>AUA1</i>	1.39	2.17
<i>RPS4a</i>	1.31	2.49	<i>MAK10</i>	0.57	2.30	<i>POC4</i>	1.16	2.17
<i>COM2</i>	1.34	2.17	<i>SSZ1</i>	0.71	2.03	<i>BUD3</i>	1.00	2.02

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<i>MRPL6</i>	0.26	2.16	<i>YPR126C</i>	1.43	2.07	<i>YGL199C</i>	0.82	1.98
<i>GEP4</i>	1.41	2.16	<i>GAS1</i>	1.41	2.07	<i>POA1</i>	0.94	1.98
<i>MME1</i>	0.92	2.15	<i>FUM1</i>	1.17	2.07	<i>FTR1</i>	0.84	1.98
<i>YDR444W</i>	0.92	2.15	<i>POS5</i>	1.01	2.06	<i>APJ1</i>	0.94	1.97
<i>LDB18</i>	0.90	2.15	<i>SPO20</i>	1.01	2.06	<i>PAL1</i>	1.05	1.97
<i>TIF3</i>	1.04	2.14	<i>TDA6</i>	1.30	2.05	<i>YGR025W</i>	0.35	1.97
<i>MUD2</i>	1.39	2.14	<i>SHH4</i>	0.16	2.05	<i>YMR058W</i>	-0.27	1.96
<i>STB4</i>	0.83	2.14	<i>YPL068C</i>	0.83	2.05	<i>RME1</i>	0.81	1.96
<i>YGL041C</i>	1.01	2.14	<i>ULA1</i>	0.82	2.05	<i>YDR467C</i>	1.00	1.96
<i>NNF2</i>	0.98	2.14	<i>YPR117W</i>	0.42	2.05	<i>YLR125W</i>	-0.26	1.96
<i>YML058C-A</i>	1.17	2.13	<i>YDR278C</i>	0.97	2.05	<i>YOR041C</i>	1.23	1.96
<i>TRM12</i>	-1.47	2.13	<i>YPL107W</i>	1.28	2.05	<i>KEL1</i>	1.06	2.09
<i>TUM1</i>	1.23	2.13	<i>SIA1</i>	1.42	2.04	<i>SOK2</i>	1.35	2.02
<i>SET4</i>	1.07	2.13	<i>DCS2</i>	1.10	2.04	<i>REC104</i>	0.96	2.02
<i>KES1</i>	1.04	2.13	<i>YPL182C</i>	0.58	2.04	<i>MAG1</i>	0.75	1.95
<i>FCY21</i>	0.84	2.13	<i>FIT2</i>	1.48	2.04	<i>MYO3</i>	0.97	1.95
<i>MAL13</i>	1.08	2.12	<i>AIM4</i>	1.39	2.04	<i>VAB2</i>	1.43	1.95
<i>VFA1</i>	1.09	2.12	<i>YML003W</i>	1.48	2.03	<i>SUR1</i>	0.84	1.94
<i>DAL7</i>	0.98	2.11	<i>YDR476C</i>	1.06	2.03	<i>LDB16</i>	1.22	1.94
<i>API2</i>	1.04	2.11	<i>ZRG8</i>	0.89	2.03	<i>NCE102</i>	0.64	1.94
<i>HNM1</i>	1.12	2.11	<i>IFM1</i>	1.30	2.02	<i>YHR022C</i>	0.19	1.94
<i>PPM1</i>	1.18	2.11	<i>TOS3</i>	0.86	2.09	<i>GLY1</i>	1.16	1.94
<i>SCP1</i>	0.54	2.11	<i>VPS15</i>	1.16	2.09	<i>HXT4</i>	1.03	1.94
<i>PMA2</i>	0.56	2.10	<i>YNL179C</i>	0.82	2.01	<i>GCV2</i>	0.37	1.93
<i>PHO8</i>	0.77	2.10	<i>RGL1</i>	0.85	2.01	<i>YNL198C</i>	1.11	1.93
<i>ERV15</i>	1.24	2.09	<i>GET4</i>	0.89	2.01	<i>DAP2</i>	1.31	1.92
<i>YDR344C</i>	0.92	2.09	<i>GGA2</i>	0.90	2.01	<i>APL5</i>	1.47	1.92
<i>RSA3</i>	0.56	2.09	<i>SNC2</i>	0.99	2.01	<i>HSP42</i>	1.30	1.92
<i>YLR171W</i>	0.50	2.09	<i>YLR415C</i>	1.49	2.00	<i>REF2</i>	1.11	1.92
<i>SLP1</i>	1.00	2.17	<i>DSF2</i>	1.23	2.00	<i>LYP1</i>	0.48	1.92
<i>PMU1</i>	0.70	2.16	<i>FIR1</i>	-0.12	2.00	<i>HXT1</i>	1.37	1.91
<i>BUD22</i>	1.52	2.09	<i>TNA1</i>	1.01	2.00	<i>YBR220C</i>	0.99	1.91
<i>YDR157W</i>	1.00	2.08	<i>SMY2</i>	0.27	1.99	<i>YDR109C</i>	1.05	1.91
<i>YGR176W</i>	0.98	2.08	<i>YGR271W</i>	0.97	1.99	<i>RTN2</i>	1.29	1.91
<i>YCL023C</i>	0.76	2.08	<i>TDA5</i>	0.99	1.99	<i>GDT1</i>	0.84	1.91
<i>MON2</i>	0.44	2.07	<i>LPD1</i>	1.44	1.99	<i>STP22</i>	0.67	1.91
<i>CUS2</i>	-2.69	2.07	<i>GVP36</i>	1.15	1.99	<i>YGL046W</i>	1.00	1.90
<i>YDL011C</i>	0.99	2.07	<i>SWI6</i>	0.82	1.98	<i>TRF5</i>	0.55	1.90
<i>SUV3</i>	0.77	2.07	<i>MAG2</i>	1.09	1.98	<i>YHR138C</i>	1.34	1.90

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<i>YGR051C</i>	1.34	2.07	<i>YMR326C</i>	0.82	1.98	<i>FAR8</i>	-0.08	1.90
<i>HXT17</i>	1.01	1.90	<i>LIF1</i>	1.17	1.85	<i>SRB5</i>	0.86	1.80
<i>RFS1</i>	1.10	1.90	<i>MPC1</i>	1.27	1.85	<i>PXP1</i>	1.38	1.80
<i>PAU2</i>	0.85	1.90	<i>CUR1</i>	1.15	1.85	<i>SYT1</i>	1.27	1.80
<i>ANR2</i>	-1.56	1.90	<i>IME1</i>	1.01	1.85	<i>RMR1</i>	1.33	1.79
<i>BIM1</i>	1.03	1.90	<i>YDR474C</i>	1.16	1.85	<i>UBP16</i>	0.82	1.79
<i>VPS73</i>	1.03	1.90	<i>OPY2</i>	0.69	1.84	<i>YDL032W</i>	0.36	1.79
<i>CTF3</i>	1.14	1.89	<i>SPI1</i>	0.88	1.84	<i>CHL1</i>	0.90	1.79
<i>YPR130C</i>	1.03	1.88	<i>VMA5</i>	0.40	1.84	<i>SHH3</i>	1.30	1.79
<i>DSE1</i>	0.72	1.88	<i>PRO2</i>	1.38	1.84	<i>YDL096C</i>	1.46	1.79
<i>CTL1</i>	-0.28	1.88	<i>YPR146C</i>	0.70	1.84	<i>MET10</i>	0.83	1.79
<i>GLR1</i>	0.24	1.88	<i>DNF3</i>	0.56	1.84	<i>AFR1</i>	0.26	1.79
<i>VPH2</i>	0.40	1.88	<i>YHR210C</i>	1.25	1.84	<i>BCK2</i>	0.98	1.79
<i>YKR011C</i>	1.03	1.88	<i>COS111</i>	1.23	1.83	<i>SAK1</i>	0.35	1.79
<i>YHR125W</i>	1.24	1.88	<i>MOD5</i>	-0.96	1.83	<i>YGR066C</i>	0.78	1.79
<i>GSY1</i>	1.16	1.88	<i>RPL20b</i>	0.09	1.83	<i>RAD6</i>	0.75	1.79
<i>HTA1</i>	-0.58	1.88	<i>RGC1</i>	0.69	1.83	<i>MEP1</i>	-1.55	1.79
<i>IRC15</i>	0.91	1.88	<i>RMD1</i>	0.59	1.83	<i>WHI4</i>	0.76	1.78
<i>YNR066C</i>	1.11	1.88	<i>NFI1</i>	0.93	1.82	<i>YDR222W</i>	-0.29	1.78
<i>JJJ3</i>	1.11	1.88	<i>ARP8</i>	0.71	1.82	<i>RSR1</i>	1.17	1.78
<i>SNF8</i>	1.04	1.88	<i>PKH3</i>	-0.82	1.82	<i>CPS1</i>	1.02	1.78
<i>ATG15</i>	0.81	1.88	<i>DDL1</i>	0.52	1.82	<i>MID1</i>	-1.58	1.78
<i>CPA1</i>	1.51	1.88	<i>PPZ1</i>	-0.45	1.82	<i>YPL062W</i>	0.92	1.78
<i>ALO1</i>	1.36	1.87	<i>MIP1</i>	0.65	1.82	<i>TPK1</i>	1.12	1.77
<i>TDA11</i>	1.05	1.87	<i>AFT2</i>	1.26	1.81	<i>CIS3</i>	0.67	1.77
<i>PET494</i>	1.11	1.87	<i>CPR5</i>	0.97	1.81	<i>VMA1</i>	1.14	1.77
<i>SMA2</i>	0.27	1.87	<i>AIM19</i>	0.57	1.81	<i>UPS2</i>	0.48	1.77
<i>LSB1</i>	1.21	1.87	<i>IES4</i>	1.38	1.81	<i>RPS21b</i>	1.36	1.77
<i>CTS2</i>	0.00	1.87	<i>DSC3</i>	1.30	1.81	<i>MDM20</i>	1.26	1.77
<i>ALD2</i>	-1.88	1.86	<i>RUD3</i>	0.98	1.81	<i>AIR2</i>	0.92	1.77
<i>YMR086C-A</i>	1.28	1.86	<i>YOR225W</i>	0.60	1.81	<i>YKL030W</i>	1.03	1.77
<i>PIN2</i>	-0.57	1.86	<i>YNR040W</i>	1.15	1.81	<i>CAF4</i>	0.93	1.77
<i>ARO2</i>	-0.14	1.86	<i>MRP51</i>	1.04	1.81	<i>CTP1</i>	1.07	1.77
<i>LEE1</i>	0.26	1.86	<i>YGL081W</i>	0.70	1.80	<i>MFA2</i>	1.03	1.77
<i>VMA3</i>	1.31	1.86	<i>MAN2</i>	0.95	1.80	<i>IRC3</i>	0.92	1.77
<i>COT1</i>	1.28	1.86	<i>LSB5</i>	1.39	1.80	<i>SDH8</i>	1.06	1.77
<i>YER084W</i>	1.08	1.86	<i>ATG31</i>	1.05	1.80	<i>DPH6</i>	1.49	1.77
<i>YCL060C</i>	1.00	1.85	<i>GRE1</i>	1.25	1.80	<i>FZO1</i>	0.62	1.77

*The values represent the log₂ (fold change) ≥ 1.5 of UPR activation in *IRE1 Δ LD* cells only, upon deletion of the respective gene (Figure 12, green). Values are calculated by the ratio of normalised GFP/mCherry signal.

Table S2. The UPR is upregulated in 777 gene deletions in *IRE1 Δ LD* cells only.

GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *	GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *	GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *
<i>RPL17b</i>	1.06	1.77	<i>YIL089W</i>	1.13	1.73	<i>TYE7</i>	0.06	1.68
<i>TOF2</i>	0.91	1.76	<i>RPA14</i>	0.08	1.72	<i>HEL2</i>	1.06	1.68
<i>LIN1</i>	0.99	1.76	<i>OCA6</i>	0.85	1.72	<i>CSC1</i>	1.42	1.68
<i>ERV25</i>	0.52	1.76	<i>YNR025C</i>	0.95	1.72	<i>YJL211C</i>	0.29	1.68
<i>RCN2</i>	1.03	1.76	<i>YDL172C</i>	0.91	1.72	<i>KEL2</i>	1.15	1.68
<i>AIM24</i>	0.98	1.76	<i>TRM7</i>	1.09	1.72	<i>HLJ1</i>	0.78	1.68
<i>COS6</i>	0.62	1.75	<i>MFT1</i>	1.47	1.72	<i>SSA4</i>	0.78	1.68
<i>ADK2</i>	1.06	1.75	<i>YDR010C</i>	1.41	1.71	<i>SAP1</i>	-0.70	1.68
<i>MRC1</i>	0.93	1.75	<i>MNN2</i>	0.79	1.71	<i>YAP3</i>	-0.21	1.68
<i>NUP42</i>	0.60	1.75	<i>RPL16b</i>	0.38	1.71	<i>VBA3</i>	1.02	1.68
<i>DUS1</i>	-0.69	1.75	<i>YFT2</i>	0.97	1.71	<i>PSK2</i>	1.42	1.68
<i>TRM9</i>	1.01	1.75	<i>LCB3</i>	0.85	1.71	<i>CYS4</i>	1.23	1.67
<i>RPS9a</i>	0.50	1.75	<i>GPD1</i>	1.38	1.71	<i>TAH1</i>	-0.52	1.67
<i>SFL1</i>	0.33	1.75	<i>ENV9</i>	0.96	1.71	<i>ISM1</i>	1.16	1.67
<i>SWE1</i>	1.42	1.75	<i>FKS3</i>	0.98	1.71	<i>PUT1</i>	1.42	1.67
<i>PPQ1</i>	0.27	1.75	<i>YCL013W</i>	0.81	1.71	<i>GIM3</i>	0.76	1.67
<i>ERP3</i>	1.11	1.75	<i>RML2</i>	0.69	1.70	<i>YLR279W</i>	-1.92	1.67
<i>YEL043W</i>	2.37	1.75	<i>REV7</i>	1.11	1.70	<i>DER1</i>	1.17	1.67
<i>MNN5</i>	0.76	1.74	<i>ARG82</i>	1.17	1.70	<i>PFK1</i>	1.25	1.67
<i>PSP1</i>	0.49	1.74	<i>YJL017W</i>	1.05	1.70	<i>FCY22</i>	1.45	1.67
<i>NAM2</i>	0.37	1.74	<i>CIS1</i>	0.96	1.70	<i>MSH1</i>	0.70	1.67
<i>ALD3</i>	1.44	1.74	<i>HIR2</i>	-0.33	1.70	<i>YGP1</i>	0.57	1.67
<i>CHZ1</i>	1.46	1.74	<i>ACE2</i>	1.08	1.70	<i>VPS33</i>	1.20	1.67
<i>VPS74</i>	0.45	1.74	<i>DXO1</i>	0.87	1.70	<i>YDR433W</i>	0.88	1.67
<i>MDG1</i>	1.06	1.74	<i>TOM7</i>	0.92	1.70	<i>RPS22a</i>	0.70	1.67
<i>RPL24b</i>	1.23	1.74	<i>LEM3</i>	0.00	1.70	<i>THR4</i>	-2.59	1.66
<i>CAR2</i>	1.09	1.74	<i>CAF40</i>	-2.08	1.70	<i>RPL35a</i>	0.74	1.66
<i>PDE2</i>	0.95	1.74	<i>YNL217W</i>	1.12	1.69	<i>SNZ2</i>	-0.17	1.66
<i>MTC2</i>	0.84	1.73	<i>MSC2</i>	1.09	1.69	<i>HNT1</i>	1.15	1.66
<i>NAT4</i>	1.16	1.73	<i>GIN4</i>	1.10	1.69	<i>YCR016W</i>	1.02	1.66
<i>DOA1</i>	1.18	1.73	<i>YNL303W</i>	0.54	1.69	<i>YER066C-A</i>	1.49	1.66
<i>YLR041W</i>	0.94	1.73	<i>YDL009C</i>	0.57	1.69	<i>CBR1</i>	0.83	1.66
<i>ADY3</i>	1.06	1.73	<i>HOS3</i>	1.10	1.69	<i>SDC1</i>	1.00	1.66
<i>RTT107</i>	1.05	1.73	<i>LDB7</i>	0.48	1.69	<i>YGL108C</i>	0.66	1.66
<i>YMR085W</i>	0.90	1.73	<i>YER034W</i>	1.35	1.69	<i>MUP1</i>	1.01	1.66
<i>MKK1</i>	1.13	1.73	<i>KAR3</i>	0.56	1.69	<i>BPT1</i>	0.33	1.66
<i>YHR079C-B</i>	1.15	1.73	<i>IMD3</i>	1.06	1.69	<i>ZWF1</i>	0.89	1.66
<i>RVS161</i>	1.00	1.73	<i>SKN7</i>	1.18	1.69	<i>RPI1</i>	0.98	1.66
<i>OAZ1</i>	1.19	1.73	<i>BIT61</i>	1.49	1.69	<i>YKR040C</i>	1.07	1.65

*The values represent the log₂ (fold change) ≥ 1.5 of UPR activation in *IRE1 Δ LD* cells only, upon deletion of the respective gene (Figure 12, green). Values are calculated by the ratio of normalised GFP/mCherry signal.

Table S2. The UPR is upregulated in 777 gene deletions in *IRE1 Δ LD* cells only.

GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *	GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *	GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *
<i>DDP1</i>	0.56	1.65	<i>GYP5</i>	0.51	1.62	<i>YNL165W</i>	1.17	1.59
<i>MPD1</i>	0.84	1.65	<i>RAD5</i>	0.95	1.62	<i>IGO1</i>	1.13	1.59
<i>YDL187C</i>	1.04	1.65	<i>SIN3</i>	1.33	1.62	<i>YBR053C</i>	1.06	1.59
<i>UGX2</i>	0.89	1.65	<i>GLN3</i>	0.66	1.62	<i>YKL118W</i>	0.55	1.59
<i>YSP3</i>	-0.50	1.65	<i>YLR006C</i>	0.96	1.62	<i>HST1</i>	0.31	1.59
<i>PCD1</i>	0.48	1.65	<i>UAF30</i>	1.56	1.62	<i>MTL1</i>	-0.92	1.59
<i>ECM23</i>	1.40	1.65	<i>PHO92</i>	0.26	1.62	<i>TDA1</i>	1.49	1.59
<i>SVP26</i>	1.12	1.65	<i>ODC1</i>	0.85	1.61	<i>YNL319W</i>	0.79	1.59
<i>SWT21</i>	1.21	1.65	<i>ASG7</i>	1.02	1.61	<i>YRF1-6</i>	-3.36	1.59
<i>SWF1</i>	0.83	1.64	<i>SET3</i>	1.19	1.61	<i>BUD9</i>	1.46	1.59
<i>YBR056W</i>	1.00	1.64	<i>YBR178W</i>	0.72	1.61	<i>HMG1</i>	1.00	1.59
<i>MSE1</i>	0.13	1.64	<i>MRX5</i>	0.85	1.61	<i>YMR153C-A</i>	0.86	1.59
<i>YJL163C</i>	1.44	1.64	<i>CIT2</i>	0.93	1.61	<i>SDH4</i>	1.05	1.59
<i>YHR045W</i>	1.05	1.64	<i>YDL186W</i>	0.56	1.60	<i>HST4</i>	0.72	1.59
<i>OMS1</i>	0.92	1.64	<i>TIM13</i>	0.62	1.60	<i>ENT5</i>	0.35	1.59
<i>YLR456W</i>	1.06	1.64	<i>VAC7</i>	0.98	1.60	<i>ADH3</i>	1.09	1.59
<i>BNR1</i>	1.03	1.64	<i>MPA43</i>	0.67	1.60	<i>MSA1</i>	0.33	1.59
<i>YPC1</i>	1.34	1.64	<i>PPH3</i>	1.06	1.60	<i>HUT1</i>	1.16	1.58
<i>MET31</i>	1.12	1.64	<i>BUD6</i>	0.30	1.60	<i>IDP1</i>	1.34	1.58
<i>RAD4</i>	0.78	1.64	<i>TGL4</i>	1.03	1.60	<i>YKR075C</i>	1.03	1.58
<i>FMP27</i>	1.09	1.64	<i>YOR302W</i>	1.97	1.60	<i>RPL27b</i>	0.64	1.58
<i>STE13</i>	0.85	1.64	<i>NPR1</i>	1.14	1.60	<i>ILM1</i>	1.27	1.58
<i>TUS1</i>	0.86	1.64	<i>LSC1</i>	1.21	1.60	<i>ZRT1</i>	1.31	1.58
<i>SHU1</i>	0.07	1.64	<i>GIP2</i>	0.98	1.60	<i>YGL132W</i>	1.16	1.58
<i>BGL2</i>	0.97	1.64	<i>ASF2</i>	1.03	1.60	<i>HIS7</i>	0.87	1.58
<i>MSS11</i>	-1.17	1.64	<i>PPG1</i>	1.04	1.60	<i>YPR1</i>	0.76	1.58
<i>PAU5</i>	1.02	1.64	<i>ACF2</i>	1.12	1.60	<i>HMI1</i>	0.81	1.58
<i>FSH2</i>	0.96	1.64	<i>ADY2</i>	1.11	1.60	<i>FRE2</i>	0.91	1.58
<i>YMR172C-A</i>	1.03	1.63	<i>YIL025C</i>	1.03	1.60	<i>YCR006C</i>	0.82	1.58
<i>MRPL39</i>	0.61	1.63	<i>GSY2</i>	-3.39	1.60	<i>SAY1</i>	0.81	1.58
<i>SPR3</i>	0.13	1.63	<i>KGD1</i>	0.93	1.60	<i>ZDS2</i>	0.68	1.58
<i>NPP1</i>	0.96	1.63	<i>REE1</i>	1.02	1.60	<i>YJL182C</i>	0.51	1.58
<i>OXF1</i>	0.96	1.63	<i>ILV6</i>	1.38	1.59	<i>MRP1</i>	0.68	1.58
<i>ENV7</i>	0.18	1.63	<i>GMC2</i>	1.16	1.59	<i>VMA21</i>	1.19	1.58
<i>HSC82</i>	1.05	1.63	<i>YLR460C</i>	0.97	1.59	<i>YNL134C</i>	0.97	1.57
<i>YSR3</i>	1.01	1.62	<i>YKL102C</i>	1.15	1.59	<i>CAJ1</i>	1.02	1.57

*The values represent the log₂ (fold change) ≥ 1.5 of UPR activation in *IRE1 Δ LD* cells only, upon deletion of the respective gene (Figure 12, green). Values are calculated by the ratio of normalised GFP/mCherry signal.

Table S2. The UPR is upregulated in 777 gene deletions in *IRE1 Δ LD* cells only.

GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *	GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *	GENE	<i>IRE1</i>	<i>IRE1ΔLD</i> *
<i>IKI3</i>	0.72	1.57	<i>RRT6</i>	0.49	1.55	<i>YCR102C</i>	1.25	1.53
<i>WTM1</i>	1.10	1.57	<i>DSE4</i>	0.95	1.55	<i>ZTA1</i>	0.92	1.53
<i>MTC6</i>	1.08	1.57	<i>YDL177C</i>	0.95	1.55	<i>CHS3</i>	1.00	1.53
<i>ARL1</i>	-0.26	1.57	<i>YDL071C</i>	0.59	1.55	<i>RPL7a</i>	1.01	1.53
<i>BUD17</i>	1.07	1.57	<i>PCI8</i>	1.01	1.55	<i>SPO77</i>	1.27	1.52
<i>CBC2</i>	0.80	1.57	<i>AAT1</i>	1.15	1.55	<i>POL4</i>	0.88	1.52
<i>SLY41</i>	0.16	1.57	<i>ADR1</i>	-1.04	1.54	<i>TYW1</i>	0.80	1.52
<i>YEL067C</i>	0.76	1.57	<i>JEN1</i>	1.03	1.54	<i>YPT31</i>	1.39	1.52
<i>CRC1</i>	-1.04	1.57	<i>THI2</i>	0.84	1.54	<i>VPS38</i>	1.29	1.52
<i>DDI3</i>	0.57	1.57	<i>ELO1</i>	0.93	1.54	<i>YLR030W</i>	0.91	1.52
<i>YOR365C</i>	0.38	1.56	<i>RCO1</i>	1.26	1.54	<i>ECM2</i>	0.97	1.52
<i>NSI1</i>	0.93	1.56	<i>YDR415C</i>	0.98	1.54	<i>LSM6</i>	1.27	1.52
<i>YIR016W</i>	0.98	1.56	<i>YNL105W</i>	1.41	1.54	<i>PUF4</i>	1.03	1.52
<i>NRK1</i>	0.97	1.56	<i>UBP7</i>	1.01	1.54	<i>MET7</i>	0.55	1.52
<i>YJL022W</i>	1.04	1.56	<i>YPL185W</i>	0.76	1.54	<i>FET5</i>	0.99	1.52
<i>GAP1</i>	0.76	1.56	<i>CRF1</i>	1.05	1.54	<i>YGR117C</i>	1.00	1.52
<i>YRO2</i>	0.94	1.56	<i>FAA4</i>	1.25	1.53	<i>MDH1</i>	0.77	1.51
<i>TRI1</i>	-0.06	1.56	<i>YPR063C</i>	1.47	1.53	<i>YKL050C</i>	-0.21	1.51
<i>EMP46</i>	-0.04	1.56	<i>MLS1</i>	1.41	1.53	<i>CTF4</i>	1.00	1.51
<i>MTE1</i>	0.79	1.56	<i>MUM3</i>	-0.81	1.53	<i>FIS1</i>	0.89	1.51
<i>ORM2</i>	0.92	1.56	<i>BOI2</i>	0.87	1.53	<i>SSN8</i>	0.78	1.51
<i>MSW1</i>	0.79	1.55	<i>GOS1</i>	1.33	1.53	<i>FZF1</i>	0.92	1.51
<i>RPS10a</i>	-0.92	1.55	<i>ACH1</i>	1.06	1.53	<i>MTC3</i>	0.83	1.51
<i>CIK1</i>	2.01	1.55	<i>YHR140W</i>	1.08	1.53	<i>YIL028W</i>	0.91	1.51
<i>YDL012C</i>	0.86	1.55	<i>YIL060W</i>	1.10	1.53	<i>YKL200C</i>	0.90	1.51
<i>SNQ2</i>	0.88	1.55	<i>IST3</i>	0.95	1.53	<i>PIR3</i>	0.37	1.51
<i>JSN1</i>	1.08	1.55	<i>YJL132W</i>	0.91	1.53	<i>SEC66</i>	1.41	1.50
<i>PNS1</i>	0.67	1.55	<i>YHR139C-A</i>	1.00	1.53	<i>RTG1</i>	1.07	1.50

*The values represent the log₂ (fold change) ≥ 1.5 of UPR activation in *IRE1 Δ LD* cells only, upon deletion of the respective gene (Figure 12, green). Values are calculated by the ratio of normalised GFP/mCherry signal.

Table S3. The UPR is upregulated in 181 gene deletions in *IRE1* and *IRE1* Δ LD cells

GENE	<i>IRE1</i> *	<i>IRE1</i> Δ LD*	GENE	<i>IRE1</i> *	<i>IRE1</i> Δ LD*	GENE	<i>IRE1</i> *	<i>IRE1</i> Δ LD*
<i>PMT2</i>	4.40	14.75	<i>SSA1</i>	3.76	3.24	<i>YJL006C</i>	1.88	2.34
<i>SCJ1</i>	3.48	14.15	<i>YOL053C-A</i>	1.65	3.22	<i>CLB6</i>	1.83	2.32
<i>AIM2</i>	2.81	11.00	<i>CIN8</i>	1.63	3.20	<i>SIP3</i>	2.70	2.29
<i>CCR4</i>	3.10	9.94	<i>YPT10</i>	1.83	3.09	<i>MTC7</i>	1.57	2.25
<i>OPI3</i>	6.16	9.58	<i>YKE2</i>	2.02	2.89	<i>YLL032C</i>	3.19	2.23
<i>VPS8</i>	2.72	9.22	<i>ARV1</i>	2.30	2.86	<i>GIS3</i>	1.57	2.23
<i>GEM1</i>	2.07	8.08	<i>YOL050C</i>	1.90	2.81	<i>ALG9</i>	1.88	2.21
<i>BUD14</i>	1.80	7.41	<i>GDH1</i>	1.99	2.78	<i>FIT3</i>	2.83	2.19
<i>SPO7</i>	2.74	6.61	<i>FMP40</i>	1.92	2.78	<i>YSY6</i>	1.89	2.16
<i>CLN3</i>	3.49	6.19	<i>LCL2</i>	2.56	2.74	<i>HOR7</i>	1.90	2.15
<i>GET1</i>	3.60	5.97	<i>HVG1</i>	1.88	2.73	<i>TPK2</i>	1.57	2.15
<i>TPO1</i>	3.15	5.93	<i>SMP1</i>	1.85	2.73	<i>AGE1</i>	2.75	2.14
<i>CCC1</i>	1.76	5.10	<i>TMN3</i>	1.68	2.69	<i>ICL1</i>	1.73	2.13
<i>FUN14</i>	2.79	4.77	<i>MET8</i>	1.71	2.69	<i>FRD1</i>	1.56	2.12
<i>MSC3</i>	1.51	4.77	<i>PMT3</i>	2.22	2.68	<i>SPT21</i>	2.35	2.12
<i>OAR1</i>	1.62	4.64	<i>RSC1</i>	1.51	2.60	<i>MEI4</i>	1.58	2.12
<i>HRD1</i>	1.91	4.57	<i>VPS29</i>	1.65	2.60	<i>HMX1</i>	1.57	2.10
<i>DEP1</i>	3.72	4.53	<i>YOR029W</i>	1.85	2.59	<i>YMR158W-A</i>	1.89	2.10
<i>NUP120</i>	2.02	4.48	<i>SNF2</i>	1.73	2.59	<i>SLT2</i>	1.76	2.09
<i>ISW1</i>	3.57	4.36	<i>YLR286C</i>	1.71	2.54	<i>YPL205C</i>	1.96	2.09
<i>FRA1</i>	2.05	4.28	<i>BEM1</i>	1.54	2.52	<i>VPS61</i>	1.63	2.08
<i>SWC5</i>	2.95	4.18	<i>YGL072C</i>	1.62	2.51	<i>YIM1</i>	2.64	2.08
<i>CTI6</i>	2.74	4.11	<i>XRS2</i>	1.75	2.50	<i>AAP1</i>	1.55	2.05
<i>ELM1</i>	1.57	4.11	<i>SPT7</i>	1.72	2.48	<i>HEH2</i>	2.01	2.04
<i>RTT109</i>	2.55	4.11	<i>STE20</i>	1.88	2.47	<i>GPA1</i>	2.92	2.03
<i>UBI4</i>	2.58	3.94	<i>AIM34</i>	1.60	2.47	<i>CHO2</i>	1.52	2.00
<i>YLR177W</i>	1.50	3.85	<i>GAS5</i>	1.69	2.46	<i>VPS72</i>	1.93	2.00
<i>SYN8</i>	4.76	3.79	<i>REV3</i>	1.62	2.44	<i>DBF2</i>	1.71	1.97
<i>PHO80</i>	2.02	3.77	<i>SLX5</i>	2.83	2.43	<i>RSN1</i>	1.52	1.96
<i>FUN26</i>	4.87	3.67	<i>MET22</i>	1.63	2.43	<i>YPL035C</i>	1.68	1.94
<i>ENV10</i>	1.99	3.56	<i>YPR148C</i>	1.63	2.43	<i>SET2</i>	1.53	1.91
<i>RBG1</i>	2.55	3.45	<i>YPL136W</i>	3.20	2.42	<i>XKS1</i>	2.73	1.91
<i>DRS2</i>	2.75	3.43	<i>YNL140C</i>	1.72	2.40	<i>ECL1</i>	1.73	1.90
<i>RNH1</i>	1.71	3.40	<i>UBC11</i>	1.99	2.39	<i>URC2</i>	2.93	1.90
<i>FUN12</i>	1.97	3.39	<i>UBR1</i>	2.11	2.38	<i>PSY3</i>	2.09	1.89
<i>YGR064W</i>	1.79	3.36	<i>YGL101W</i>	5.11	2.37	<i>SEC22</i>	1.78	1.89
<i>CLB4</i>	2.46	3.35	<i>SPT4</i>	1.92	2.37	<i>TLG2</i>	2.23	1.87

*The values represent the log₂ (fold change) ≥ 1.5 of UPR activation in *IRE1* and *IRE1* Δ LD cells, upon deletion of the respective gene (Figure 12, red). Values are calculated by the ratio of normalised GFP/mCherry signal.

Table S3. The UPR is upregulated in 181 gene deletions in *IRE1* and *IRE1* Δ *LD* cells

GENE	<i>IRE1</i> *	<i>IRE1</i> Δ <i>LD</i> *	GENE	<i>IRE1</i> *	<i>IRE1</i> Δ <i>LD</i> *	GENE	<i>IRE1</i> *	<i>IRE1</i> Δ <i>LD</i> *
<i>AVL9</i>	1.79	1.85	<i>CHS5</i>	1.83	1.71	<i>MEC3</i>	1.92	1.57
<i>EDC3</i>	2.00	1.85	<i>ISN1</i>	1.71	1.71	<i>YGL140C</i>	2.01	1.57
<i>PXA1</i>	1.54	1.84	<i>TOR1</i>	2.22	1.71	<i>SSE1</i>	1.88	1.56
<i>YLL017W</i>	1.66	1.84	<i>RGP1</i>	1.71	1.69	<i>PKH1</i>	1.83	1.56
<i>LCB4</i>	2.00	1.83	<i>YPK2</i>	2.46	1.68	<i>YDR521W</i>	1.90	1.56
<i>MCT1</i>	1.94	1.83	<i>CYC3</i>	3.09	1.68	<i>PCT1</i>	1.61	1.55
<i>MRX4</i>	1.58	1.83	<i>HMF1</i>	1.56	1.68	<i>RCH1</i>	1.55	1.55
<i>ORT1</i>	2.08	1.83	<i>GCV3</i>	2.46	1.67	<i>BMH2</i>	2.07	1.55
<i>SER2</i>	1.83	1.82	<i>SCD6</i>	1.97	1.67	<i>ATG40</i>	1.94	1.55
<i>YDR290W</i>	1.61	1.82	<i>RGS2</i>	1.62	1.67	<i>SMI1</i>	1.71	1.54
<i>PEX9</i>	2.80	1.82	<i>CLU1</i>	1.89	1.66	<i>JEM1</i>	1.58	1.54
<i>SPC2</i>	4.17	1.80	<i>YOL153C</i>	1.86	1.65	<i>YMR155W</i>	1.98	1.53
<i>PNP1</i>	1.65	1.80	<i>EMP24</i>	1.62	1.64	<i>YML002W</i>	1.77	1.52
<i>GRX1</i>	1.55	1.80	<i>CTF18</i>	2.35	1.64	<i>PRY3</i>	1.86	1.52
<i>CLA4</i>	1.58	1.79	<i>MRPL25</i>	2.04	1.63	<i>PFA4</i>	1.69	1.52
<i>REC8</i>	2.59	1.79	<i>YIR043C</i>	1.84	1.63	<i>BUD31</i>	1.67	1.51
<i>VRP1</i>	1.97	1.79	<i>YGR291C</i>	1.57	1.62	<i>APA2</i>	2.19	1.51
<i>PEF1</i>	1.72	1.78	<i>YRM1</i>	2.25	1.59	<i>HRD3</i>	2.25	1.51
<i>DOC1</i>	2.52	1.76	<i>HTB2</i>	1.54	1.58	<i>YLR345W</i>	3.31	1.51
<i>HUA2</i>	1.61	1.76	<i>YPT6</i>	1.56	1.58	<i>SHE10</i>	2.05	1.51
<i>VPS63</i>	2.05	1.74	<i>PMT6</i>	2.91	1.58	<i>HMG2</i>	1.59	1.50
<i>PTM1</i>	2.28	1.72	<i>PEP4</i>	2.05	1.57			
<i>RRT2</i>	1.85	1.71	<i>YOR015W</i>	1.73	1.57			

*The values represent the log₂ (fold change) ≥ 1.5 of UPR activation in *IRE1* and *IRE1* Δ *LD* cells, upon deletion of the respective gene (Figure 12, red). Values are calculated by the ratio of normalised GFP/mCherry signal.

Table S4: List of genes upregulated in different subsets of proteotoxic and/or lipid bilayer stress in cells expressing Ire1 and/or Ire1 Δ LD (Refer to Figure 39, 40).

<i>ire1Δopi3Δ IRE1</i> n <i>ire1Δopi3Δ IRE1ΔLD</i>	<i>ire1Δ IRE1 + DTT</i> n <i>ire1Δopi3Δ IRE1</i> n <i>ire1Δopi3Δ IRE1ΔLD</i>	<i>ire1Δopi3Δ IRE1ΔLD</i>	<i>ire1Δ IRE1 + DTT</i> n <i>ire1Δopi3Δ IRE1</i>	<i>ire1Δopi3Δ IRE1</i>
RNP1	ULI1	RRT5	IRC4	FRE3
PIR3	SIL1	HIS3	URH1	SUL1
GAC1	YBL065W	YGR035C	GIP1	YHR071C-A
DSF1	MCD4	YGR160W	SPP382	YPR150W
PHO5	DOG1	IDH1	MPA43	GUD1
MTC7	ADD37	GSM1	HLR1	RCK1
YLR312C	DER1	SER3	YDR210W	YBR259W
YCL048W-A	JEM1	YIL169C	PMT5	SEC24
YBL048W	LHS1	PET18	HEM15	KIC1
MOH1	YDR320W-B	COX1	YGR122C-A	YGR149W
MAL12	DAN4	SKM1	YLR126C	ASP3-4
YPR160W-A	DOG2	HXT4	GWT1	CCP1
ADH7	YPL247C	YLL020C	TOS3	AMD2
DDI3	GSY1	ODC1	NBP1	ASP3-1
MAL32	FPR2	COQ9	SEC59	INP2
DDI2	INO1	YGR109W-A	YMR187C	REE1
SFC1	YGR039W	REC102	YBR137W	ASP3-3
CSR2	MGR1	MAL31	YIL102C-A	YRB2
DAL4	YOL155W-A	YAL037C-A	LDB17	PTI1
FBP1	YHR165W-A	BNR1	PMI40	ASP3-2
YGR174W-A	YGR259C	PRR2	SEC62	YCL041C
CYC7	COQ6	FIT3	HNT1	TYR1
ALP1	THI20	SDH2	CWC25	OM14
IDP2	VTC3	YDR357C	SOH1	DPM1
SRX1	YIL108W	YMR007W	ERP1	YFR016C
YGR153W	YPL136W	HPF1	DFR1	CDC23
SET4	CLP1	MSF1	TPO5	DAL80
MCH5	PAU22	AHC1	YPL162C	BIR1
YNR065C	PAU21	ANB1	FCY21	FUN19
YBR201C-A	PAU15	FLO10	TRK2	SOM1
PDH1	PAU19	YNL058C	YDR230W	SNT2
CRG1	SWA2	ACH1	YDR338C	YPR064W
SPS22	VHS3	RPM2	ADD66	MUK1

In the overlap of *IRE1 ire1 Δ opi3 Δ* and *IRE1 Δ LD ire1 Δ opi3 Δ* cells, 214 (red) genes are positively upregulated. In *IRE1 ire1 Δ opi3 Δ* or *IRE1 Δ LD ire1 Δ opi3 Δ* cells, 92 (orange) or 56 (blue) genes are upregulated respectively. The overlap of DTT treated *IRE1 ire1 Δ* cells to *IRE1 ire1 Δ opi3 Δ* and *IRE1 Δ LD ire1 Δ opi3 Δ* cells show an upregulated 77 (purple) genes, encompassing known gene targets of the UPR. The overlap of DTT treated *IRE1 ire1 Δ* cells and *IRE1 ire1 Δ opi3 Δ* cells show an upregulation of 73 (green) genes in response to proteotoxic stress and lipid bilayer stress.

Table S4: List of genes upregulated in different subsets of proteotoxic and/or lipid bilayer stress expressing Ire1 and/or Ire1ΔLD (Refer to Figure 39, 40).

<i>ire1Δopi3Δ IRE1</i> ∩ <i>ire1Δopi3ΔIRE1ΔLD</i>	<i>ire1Δ IRE1 + DTT</i> ∩ <i>ire1Δopi3Δ IRE1</i> ∩ <i>ire1Δopi3ΔIRE1ΔLD</i>	<i>ire1Δopi3Δ</i> <i>IRE1ΔLD</i>	<i>ire1Δ IRE1 + DTT</i> ∩ <i>ire1Δopi3Δ IRE1</i>	<i>ire1Δopi3Δ</i> <i>IRE1</i>
YBR178W	LSC2	RNR4	SSY1	YLR173W
YJL133C-A	HUG1	RIM4	TSA1	YMR181C
SPS1	YGR161W-C	YBR032W	PRM2	YBL100W-C
YPR015C	RRI2	RNR2	MUD2	RMD5
ALD4	YLR446W	SDH4	TMA64	YER152C
GDH3	THI12	YAP6	VAC7	LEU1
PCA1	THI11	MLH3	PYK2	BFA1
SRT1	MMS21	HER1	RPR2	RRD1
YPR127W	RTS3	PPZ1	YJL213W	PCL7
PFK26	THI5	YPL272C	ACB1	CHS7
RNR3	YMR291W	DFG5	LSM8	MCT1
YBR071W	RIB1	KGD1	GPI17	SIM1
MTH1	YOR072W	BET3	YAL016C-A	GLG2
PAU23	YOL159C-A	YKL162C	MED11	UGA4
ADH2	TPO4	AI2	FOX2	YAR023C
DAK2	MLS1	SHC1	BFR1	CBK1
GDE1	PEX27	CDC28	ARF2	YGR201C
PTP2	ROM1	ROD1	YPL191C	TPP1
IML2	YKL187C	TAM41	YER067W	AST2
YDL057W	GIT1	YCL049C	YPL150W	IME1
COX2	PBI2	DBF2	TFC6	YMR317W
FLO1	MYO3	YMR141C	MAD2	AAD16
YJL132W	ESC1	ECM13	SEC12	ECM38
YNR066C	PCK1	ROX3	HSF1	ITR1
FBP26	THI72	ADE16	YIR016W	PSK2
DMC1	OYE2	YLR352W	CYC2	INO4
HXT7	YOR192C-C	ARE1	UBR2	MNN4
GAL2	GDH2		ZAP1	YEL057C
YPL088W	GAL4		MUS81	TC21003
PST1	YLR338W		TAF7	PEP8
HXT6	STB3		YAL064W	YLR162W
YOR338W	YMR118C		YPR123C	ASK10
SPO11	YMR315W		DAP1	GTO1
YLR415C	ARA2		YEL020C	PHR1
IRC20	ATG3		NRG2	MGA2

In the overlap of *IRE1 ire1Δopi3Δ* and *IRE1ΔLD ire1Δopi3Δ* cells, 214 (red) genes are positively upregulated. In *IRE1 ire1Δopi3Δ* or *IRE1ΔLD ire1Δopi3Δ* cells, 92 (orange) or 56 (blue) genes are upregulated respectively. The overlap of DTT treated *IRE1 ire1Δ* cells to *IRE1 ire1Δopi3Δ* and *IRE1ΔLD ire1Δopi3Δ* cells show an upregulated 77 (purple) genes, encompassing known gene targets of the UPR. The overlap of DTT treated *IRE1 ire1Δ* cells and *IRE1 ire1Δopi3Δ* cells show an upregulation of 73 (green) genes in response to proteotoxic stress and lipid bilayer stress.

Table S4: List of genes upregulated in different subsets of proteotoxic and/or lipid bilayer stress expressing Ire1 and/or Ire1 Δ LD (Refer to Figure 39, 40).

<i>ire1Δopi3Δ IRE1</i> ∩ <i>ire1Δopi3ΔIRE1ΔLD</i>	<i>ire1Δ IRE1 + DTT</i> ∩ <i>ire1Δopi3Δ IRE1</i> ∩ <i>ire1Δopi3ΔIRE1ΔLD</i>	<i>ire1Δopi3Δ IRE1ΔLD</i>	<i>ire1Δ IRE1 + DTT</i> ∩ <i>ire1Δopi3Δ IRE1</i>	<i>ire1Δopi3Δ IRE1</i>
<i>PDE1</i>	<i>SNZ3</i>		<i>MSS11</i>	<i>BCY1</i>
<i>YPL067C</i>	<i>ICL1</i>		<i>YDL211C</i>	<i>YCR050C</i>
<i>URA10</i>	<i>SNZ2</i>		<i>YJR114W</i>	<i>YJL045W</i>
<i>ACA1</i>	<i>PAU7</i>		<i>YJL216C</i>	<i>HTD2</i>
<i>Q0255</i>	<i>YDL218W</i>		<i>STE11</i>	<i>IRC3</i>
<i>CRC1</i>	<i>ECM21</i>		<i>PEX1</i>	<i>YLR361C-A</i>
<i>HAL1</i>	<i>ICL2</i>		<i>SFB3</i>	<i>TAP42</i>
<i>SRL3</i>	<i>YKL071W</i>		<i>YFR018C</i>	<i>YIL014C-A</i>
<i>MPC54</i>	<i>PSY3</i>			<i>RTT101</i>
<i>YGL015C</i>				<i>FMP21</i>
<i>YDR374W-A</i>				<i>YMR316C-B</i>
<i>YMR315W-A</i>				<i>MATALPHA1</i>
<i>DIT1</i>				<i>CDD1</i>
<i>RIM15</i>				<i>YJL206C</i>
<i>PKH2</i>				<i>PDR12</i>
<i>QDR2</i>				<i>AVO2</i>
<i>YCR099C</i>				<i>ETP1</i>
<i>HXT2</i>				<i>SEC16</i>
<i>HEF3</i>				<i>YGL185C</i>
<i>AI5_BETA</i>				<i>GAS5</i>
<i>YPR077C</i>				<i>BIO4</i>
<i>YCL012C</i>				<i>WTM1</i>
<i>YLR030W</i>				<i>HMLALPHA1</i>
<i>REC8</i>				<i>CHO2</i>
<i>CHS6</i>				<i>VPS51</i>
<i>SUE1</i>				<i>YNL193W</i>
<i>YLR225C</i>				<i>RRT13</i>
<i>PCS60</i>				<i>PIG2</i>
<i>YJL142C</i>				<i>VPS8</i>
<i>YKR015C</i>				<i>YMR194C-B</i>
<i>COX3</i>				<i>SAE2</i>
<i>YMR085W</i>				<i>YHR045W</i>
<i>TAD2</i>				<i>ALG14</i>

In the overlap of *IRE1 ire1 Δ opi3 Δ* and *IRE1 Δ LD ire1 Δ opi3 Δ* cells, 214 (red) genes are positively upregulated. In *IRE1 ire1 Δ opi3 Δ* or *IRE1 Δ LD ire1 Δ opi3 Δ* cells, 92 (orange) or 56 (blue) genes are upregulated respectively. The overlap of DTT treated *IRE1 ire1 Δ* cells to *IRE1 ire1 Δ opi3 Δ* and *IRE1 Δ LD ire1 Δ opi3 Δ* cells show an upregulated 77 (purple) genes, encompassing known gene targets of the UPR. The overlap of DTT treated *IRE1 ire1 Δ* cells and *IRE1 ire1 Δ opi3 Δ* cells show an upregulation of 73 (green) genes in response to proteotoxic stress and lipid bilayer stress.

Table S4: List of genes upregulated in different subsets of proteotoxic and/or lipid bilayer stress expressing Ire1 and/or Ire1 Δ LD (Refer to Figure 39, 40).

<i>ire1Δopi3Δ IRE1</i> $\cap$ <i>ire1Δopi3ΔIRE1ΔLD</i>	<i>ire1Δ IRE1 + DTT</i> $\cap$ <i>ire1Δopi3Δ IRE1</i> $\cap$ <i>ire1Δopi3ΔIRE1ΔLD</i>	<i>ire1Δopi3Δ IRE1ΔLD</i>	<i>ire1Δ IRE1 + DTT</i> $\cap$ <i>ire1Δopi3Δ IRE1</i>	<i>ire1Δopi3Δ IRE1</i>
YJR008W				
YHL012W				
YDL119C				
PXA2				
YKL102C				
ATP8				
YOL107W				
CTL1				
YCR100C				
NDT80				
YOR032W-A				
SPO1				
MAL33				
GFA1				
YOR114W				
THI2				
YLR281C				
YIL024C				
YER078W-A				
VAR1				
AMA1				
YAK1				
SMP1				
YEH1				
SPO20				
IDP3				
UBP9				
IME2				
MAL11				
YOL159C				
YKE4				
COS111				
CTA1				
PET10				
HPA2				

In the overlap of *IRE1 ire1 Δ opi3 Δ* and *IRE1 Δ LD ire1 Δ opi3 Δ* cells, 214 (red) genes are positively upregulated. In *IRE1 ire1 Δ opi3 Δ* or *IRE1 Δ LD ire1 Δ opi3 Δ* cells, 92 (orange) or 56 (blue) genes are upregulated respectively. The overlap of DTT treated *IRE1 ire1 Δ* cells to *IRE1 ire1 Δ opi3 Δ* and *IRE1 Δ LD ire1 Δ opi3 Δ* cells show an upregulated 77 (purple) genes, encompassing known gene targets of the UPR. The overlap of DTT treated *IRE1 ire1 Δ* cells and *IRE1 ire1 Δ opi3 Δ* cells show an upregulation of 73 (green) genes in response to proteotoxic stress and lipid bilayer stress.

Table S4: List of genes upregulated in different subsets of proteotoxic and/or lipid bilayer stress expressing Ire1 and/or Ire1 Δ LD (Refer to Figure 39, 40).

<i>ire1Δopi3Δ IRE1</i> ∩ <i>ire1Δopi3ΔIRE1ΔLD</i>	<i>ire1Δ IRE1 + DTT</i> ∩ <i>ire1Δopi3Δ IRE1</i> ∩ <i>ire1Δopi3ΔIRE1ΔLD</i>	<i>ire1Δopi3Δ IRE1ΔLD</i>	<i>ire1Δ IRE1 + DTT</i> ∩ <i>ire1Δopi3Δ IRE1</i>	<i>ire1Δopi3Δ IRE1</i>
<i>PSR2</i>				
<i>SPO13</i>				
<i>YHL008C</i>				
<i>CIS3</i>				
<i>YCR101C</i>				
<i>SED1</i>				
<i>PTC6</i>				
<i>LCB5</i>				
<i>ZRG8</i>				
<i>YER077C</i>				
<i>YHR212C</i>				
<i>YML131W</i>				
<i>YMR103C</i>				
<i>YKL162C-A</i>				
<i>DIT2</i>				
<i>YGR168C</i>				
<i>YOL106W</i>				
<i>YGL034C</i>				
<i>TRX3</i>				
<i>YDR010C</i>				
<i>YSP3</i>				
<i>YHL048C-A</i>				
<i>AI4</i>				
<i>IPK1</i>				
<i>YNL146W</i>				
<i>YJR061W</i>				
<i>MOD5</i>				
<i>YPK2</i>				
<i>ATG11</i>				
<i>YMR114C</i>				
<i>YAR060C</i>				
<i>MSB3</i>				
<i>PPZ2</i>				
<i>RIM21</i>				

In the overlap of *IRE1 ire1 Δ opi3 Δ* and *IRE1 Δ LD ire1 Δ opi3 Δ* cells, 214 (red) genes are positively upregulated. In *IRE1 ire1 Δ opi3 Δ* or *IRE1 Δ LD ire1 Δ opi3 Δ* cells, 92 (orange) or 56 (blue) genes are upregulated respectively. The overlap of DTT treated *IRE1 ire1 Δ* cells to *IRE1 ire1 Δ opi3 Δ* and *IRE1 Δ LD ire1 Δ opi3 Δ* cells show an upregulated 77 (purple) genes, encompassing known gene targets of the UPR. The overlap of DTT treated *IRE1 ire1 Δ* cells and *IRE1 ire1 Δ opi3 Δ* cells show an upregulation of 73 (green) genes in response to proteotoxic stress and lipid bilayer stress.

Table S4: List of genes upregulated in different subsets of proteotoxic and/or lipid bilayer stress expressing Ire1 and/or Ire1 Δ LD (Refer to Figure 39, 40).

<i>ire1Δopi3Δ IRE1</i> ∩ <i>ire1Δopi3ΔIRE1ΔLD</i>	<i>ire1Δ IRE1 + DTT</i> ∩ <i>ire1Δopi3Δ IRE1</i> ∩ <i>ire1Δopi3ΔIRE1ΔLD</i>	<i>ire1Δopi3Δ IRE1ΔLD</i>	<i>ire1Δ IRE1 + DTT</i> ∩ <i>ire1Δopi3Δ IRE1</i>	<i>ire1Δopi3Δ IRE1</i>
YGL036W				
KRE11				
SNA3				
STU1				
YJL070C				
UPC2				
THI73				
SIP4				
SAG1				
AAD6				
BIO3				
PLB1				
YKR005C				
ATG4				
SSK22				
YBR230W-A				
NAB6				
YER158C				
YNL195C				
RSF1				
YMR295C				
YML116W-A				
SPO22				
YJR149W				
ATG32				
AFR1				
PPQ1				
MEC1				
SPT14				
YIP3				
YKR096W				
ACS1				
YBR225W				
YKL171W				

In the overlap of *IRE1 ire1 Δ opi3 Δ* and *IRE1 Δ LD ire1 Δ opi3 Δ* cells, 214 (red) genes are positively upregulated. In *IRE1 ire1 Δ opi3 Δ* or *IRE1 Δ LD ire1 Δ opi3 Δ* cells, 92 (orange) or 56 (blue) genes are upregulated respectively. The overlap of DTT treated *IRE1 ire1 Δ* cells to *IRE1 ire1 Δ opi3 Δ* and *IRE1 Δ LD ire1 Δ opi3 Δ* cells show an upregulated 77 (purple) genes, encompassing known gene targets of the UPR. The overlap of DTT treated *IRE1 ire1 Δ* cells and *IRE1 ire1 Δ opi3 Δ* cells show an upregulation of 73 (green) genes in response to proteotoxic stress and lipid bilayer stress.

Table S4: List of genes upregulated in different subsets of proteotoxic and/or lipid bilayer stress expressing Ire1 and/or Ire1 Δ LD (Refer to Figure 39, 40).

<i>ire1Δopi3Δ IRE1</i> $\cap$ <i>ire1Δopi3ΔIRE1ΔLD</i>	<i>ire1Δ IRE1 + DTT</i> $\cap$ <i>ire1Δopi3Δ IRE1</i> $\cap$ <i>ire1Δopi3ΔIRE1ΔLD</i>	<i>ire1Δopi3Δ IRE1ΔLD</i>	<i>ire1Δ IRE1 + DTT</i> $\cap$ <i>ire1Δopi3Δ IRE1</i>	<i>ire1Δopi3Δ IRE1</i>
YMR124W				
TIP41				
FLC2				
FLO5				
YGL007W				
OAZ1				
YOL036W				
YEL073C				
MXR2				
FRE6				

In the overlap of *IRE1 ire1 Δ opi3 Δ* and *IRE1 Δ LD ire1 Δ opi3 Δ* cells, 214 (red) genes are positively upregulated. In *IRE1 ire1 Δ opi3 Δ* or *IRE1 Δ LD ire1 Δ opi3 Δ* cells, 92 (orange) or 56 (blue) genes are upregulated respectively. The overlap of DTT treated *IRE1 ire1 Δ* cells to *IRE1 ire1 Δ opi3 Δ* and *IRE1 Δ LD ire1 Δ opi3 Δ* cells show an upregulated 77 (purple) genes, encompassing known gene targets of the UPR. The overlap of DTT treated *IRE1 ire1 Δ* cells and *IRE1 ire1 Δ opi3 Δ* cells show an upregulation of 73 (green) genes in response to proteotoxic stress and lipid bilayer stress.

Figure S1: Uncropped gel image corresponding to immunoblot of Figure 18 of the thesis.

Blots of the left and right are 2 individual gels separated by the 800 nm and 680 nm channels using the LI-COR Odyssey CLx Near-Infrared Fluorescence Imaging. Top: 800 nm channel for the detection of mouse α -HA for Ire1-HA and Δ LD-HA. Bottom: 680 nm channel for the detection of rabbit Kar2 and Sec61. The 800 nm and 680 nm channels have been separated because Δ LD-HA and Kar2 are 75 kDa. * denotes the non-specific band. The dotted lines represent the area of the gel cropped to make Figure 18.

Figure S2: Uncropped gel image corresponding to the 8% acrylamide gel for the resolution of small DNA fragments of Figure 20A of the thesis. The dotted lines represent the area of the gel cropped to make Figure 20A.

Figure S3: Uncropped gel image corresponding to the immunoblot of Figure 23B of the thesis. The blot is an individual gel visualised by the 800 nm channel using the LICOR Odyssey CLx Near-Infrared Fluorescence Imaging. The dotted lines represent the area of the gel cropped to make the Figure 23B.

Figure S4: Uncropped gel image corresponding to the immunoblot of Figure 25A of the thesis. The blot is an individual gel visualised by the 680 nm channel using the LI-COR Odyssey CLx Near-Infrared Fluorescence Imaging. The dotted lines represent the area of the gel cropped to make Figure 25A.

Figure S5: Uncropped gel image corresponding to the 8% acrylamide gel for the resolution of small DNA fragments of Figure 31 of the thesis. The dotted lines represent the area of the gel cropped to make the Figure 31.

Figure S6: Uncropped gel image corresponding to the 8% acrylamide gel for the resolution of small DNA fragments of Figure 35 of the thesis. The dotted lines represent the area of the gel cropped to make Figure 35.

Figure S7: Uncropped gel image corresponding to the immunoblot of Figure 43 of the thesis. The blot is an individual gel visualised by the 800 nm channel using the LI-COR Odyssey CLx Near-Infrared Fluorescence Imaging. The dotted lines represent the area of the gel cropped to make the Figure 43.

END