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Hyperactive TORC1 Sensitizes Yeast Cells to Endoplasmic Reticulum Stress by Compromising Cell Wall Integrity

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Abstract

The disruption of protein folding homeostasis in the endoplasmic reticulum (ER) results in an accumulation of toxic misfolded proteins and activates a network of signaling events collectively known as the unfolded protein response (UPR). While UPR activation upon ER stress is well characterized, how other signaling pathways integrate into the ER proteostasis network is unclear. Here, I sought to investigate how the target of rapamycin complex 1 (TORC1) signaling cascade acts in parallel with the UPR to regulate ER stress sensitivity. Using *S. cerevisiae*, I found that TORC1 signaling is attenuated during ER stress and constitutive activation of TORC1 increases sensitivity to ER stressors such as tunicamycin and inositol deprivation. This phenotype is independent of the UPR. Transcriptome analysis revealed that TORC1 hyperactivation results in cell wall remodeling. Conversely, hyperactive TORC1 sensitizes cells to cell wall stressors, including the antifungal caspofungin. Elucidating the crosstalk between the UPR, cell wall integrity, and TORC1 signaling may uncover new paradigms through which the response to protein misfolding is regulated, and thus have crucial implications for the development of novel therapeutics against pathogenic fungal infections.

Keywords

Endoplasmic reticulum stress, TORC1, UPR, cell wall integrity, fungal pathogenesis

Co-Authorship Statement

I performed all of the experiments in this thesis, with the exception of the microarray experiments, which were conducted by David Carter at the London Regional Genomics Center.

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ER	Endoplasmic Reticulum
UPR	Unfolded Protein Response
TORC1	Target of Rapamycin Complex 1
SRP	Signal Recognition Particle
ERAD	Endoplasmic Reticulum Associated Degradation
DTT	Dithiothreitol
Tm	Tunicamycin
PA	Phosphatidic Acid
CWI	Cell Wall Integrity
Ire1	Inositol Requiring Enzyme 1
PERK	Pancreatic ER eIF2α kinase
ATF6	Activating Transcription Factor 6
BiP	Binding Immunoglobulin Protein
XBP1	X box Binding Protein 1
UPRE	Unfolded Protein Response Element
RIDD	Regulated Ire1 Dependent Decay
IFI	Invasive Fungal Infections
YPD	Yeast Extract Peptone Dextrose
SC	Synthetic Complete (media)
GSR	General Stress Response

GEF	GTP exchange factor
HD	Huntington’s Disease
PolyQ	Polyglutamine
Htt	Huntingtin (protein)
TAC	Transcriptome analysis console

Chapter 1

1 Literature Review

1.1 The Role of ER Homeostasis in Human Pathology

A number of environmental and genetic conditions can impair protein-folding fidelity in the endoplasmic reticulum (ER), leading to a build-up of misfolded proteins within the organelle– a condition known as ER stress[1–3]. To restore protein-folding homeostasis, the cell activates a network of intracellular signaling events known as the unfolded protein response (UPR)[2–4]. Chronic ER stress and malfunctions in UPR signaling have emerged as key contributors to a number of human diseases such as neurodegeneration [5,6], cancer[7–9], and pathogenic fungal infections[10–13]; however, what remains unclear is how these signaling pathways mediate disease progression.

A hallmark of the pathology of neurodegeneration is the accumulation of protein aggregates and misfolded proteins within neurons and surrounding cells. For example, in Parkinson's disease, ubiquitinated protein aggregates of α-synuclein form characteristic Lewy bodies[14]. Additionally, Alzheimer's disease is characterized by both extracellular deposits of the amyloid-β protein, as well as intracellular deposits of tau protein[15]. As a whole, the common theme between these neurological diseases is that the accumulation of misfolded proteins disrupts protein-folding homeostasis in the ER. While ER stress-driven neurotoxicity is well established, what remains to be understood are the signaling pathways and mechanisms that mediate these diseases.

Similarly, the role of ER stress in cancer is well established, but poorly understood[16,17]. Tumor cells often grow in unfavourable conditions such as hypoxia, inadequate nutrition, and oxidative stress – all of which compromise protein folding within the ER[16,17]. As a result, sustained activation of UPR signaling and increased expression of downstream UPR targets are prevalent in a wide array of human tumors including glioblastomas and carcinomas of the breast, stomach, and liver[7–9]. Despite the vast amount of evidence implicating ER stress and UPR activation in cancer, how these processes inhibit or promote tumor growth, remains to be understood.

Additionally, pathogenic fungi, such as *Aspergillus fumigatus* – the leading agent of fungal infections in immunocompromised patients, rely heavily on the secretory pathway to mediate cell wall integrity and enzyme secretion during infection[18]. Recent studies

suggest a direct role for the ER in facilitating essential fungal traits such as biofilm formation and virulence[10,12,18]. Furthermore, the inability of pathogenic fungi to cause disease when their ER stress responses[11,18] are impaired suggests that targeting molecules that disrupt these stress pathways could be useful in developing novel anti-fungal therapies. Therefore, elucidating the signaling pathways that interact with the UPR under conditions of ER stress will facilitate our understanding of ER-stress related disease progression.

1.2 Secretory Pathway Homeostasis

The ER is a membrane-bound organelle responsible for the synthesis, post-translational modification, folding, and quality control of secretory proteins[2,19,20]. Secretory protein translation is initiated by cytosolic ribosomes; however, the emergence of a signal peptide allows the polypeptide to be recognized by a signal recognition particle (SRP), which directs the ribosome to the ER[21,22]. Once the ribosome becomes bound to the ER membrane, polypeptide synthesis continues, and the polypeptide enters the ER lumen co-translationally. The environment of the ER lumen is highly specialized for protein folding; not only does the oxidizing potential support disulphide bond formation, but the high concentration of chaperone proteins also helps to minimize protein aggregation and facilitate native structure formation[20,23,24]. While the processes of protein folding and maturation are assisted, they are also sensitive to changes in ER homeostasis, such as altered metabolic states, increases in protein synthesis, and the expression of misfolded proteins[25]. Conditions that perturb ER homeostasis generate a state known as ER stress, which can compromise cell integrity due to the accumulation of misfolded proteins[2,4,26]. Therefore, to ensure protein-folding fidelity, cells have evolved an ER quality control mechanism, ER-associated degradation (ERAD), that ensures that only properly folded proteins are trafficked to the Golgi apparatus, and that misfolded proteins are targeted to the proteasome for degradation[27–30]. If, however, the accumulation of misfolded proteins exceeds the capacity of the ER quality control machinery, the cell enters a state of stress and elicits a network of intracellular signaling and transcriptional events that are collectively known as the unfolded protein response (UPR)[2,4].

1.3 Endoplasmic Reticulum Stress

Depending on the physiological state of the cell, the flux of polypeptides into the ER can be highly dynamic. Therefore, to preserve protein-folding fidelity, cells adjust the protein-folding capacity of the ER to meet cellular demands. However, ER homeostasis can be perturbed by both physiological and pathological conditions such as nutrient deprivation, high protein demand, or mutant protein expression – all of which can result in an accumulation of unfolded or misfolded proteins in the ER lumen, a condition termed ER stress[31–33]. Additionally, several chemicals induce ER stress in a cell culture system, including tunicamycin and dithiothreitol (DTT). Tunicamycin blocks the initial step of glycoprotein biosynthesis in the ER by inhibiting UDP-GlcNAc-phosphate transferase[34]. Therefore, treatment with tunicamycin causes an accumulation of misfolded glycoproteins in the ER, consequently leading to UPR activation. DTT is a potent reducing agent that disrupts the formation of disulfide bonds between cysteine residues and causes unfolded proteins to accumulate in the ER[35–37].

Changes in lipid metabolism also activate the UPR, independently of the response caused by an accumulation of misfolded proteins in the ER [38,39]. The observation that the ER protein folding sensor, Ire1, lacking its luminal misfolded protein-sensing domain, was activated in yeast deprived of lipid precursors provides direct evidence that lipids activate ER stress response programs independently of their effects on the misfolded protein burden in the ER lumen [40,41]. Furthermore, in yeast studies, where cells were depleted of phospholipid building blocks, chaperone protein mobility was significantly increased compared to ER stress conditions where the accumulation of misfolded proteins in the ER lumen slowed down chaperone protein mobility[38]. Taken together, these observations suggest that altering lipid metabolism activates the UPR independently of unfolded protein levels, implying that multiple modes of UPR activation may exist.

In particular, the phospholipid building block, inositol, plays an essential role in the interplay between lipid metabolism and ER stress signaling[2,42]. Perturbations in inositol metabolism are associated with the activation of several key stress response pathways such as the UPR and cell wall integrity (CWI) pathways[42–44]. In fact, early studies showing that mutations in the UPR pathway confer inositol auxotrophy, highlight the

notion that stress response signaling is activated by inositol starvation. The depletion of inositol triggers the ER stress sensor, Ire1, which induces the transcription of inositol biosynthetic genes such as *INO1*, the enzyme that catalyzes the rate limiting step of inositol synthesis[3,45]. The regulation of *INO1* transcription, itself, is mediated by the repressor protein, Opi1 (Fig. 1.1). Normally, under conditions of high inositol, Opi1 translocates from the ER to the nucleus, where it represses *INO1* transcription[42]. On the other hand, low concentrations of inositol prevent Opi1 translocation and thus allow expression of *INO1*[42]. While it is unclear how exactly inositol deprivation triggers ER stress, some studies postulate that it triggers the UPR by either causing changes in the lipid composition of the ER membrane[46] or by impairing membrane trafficking[44,47].

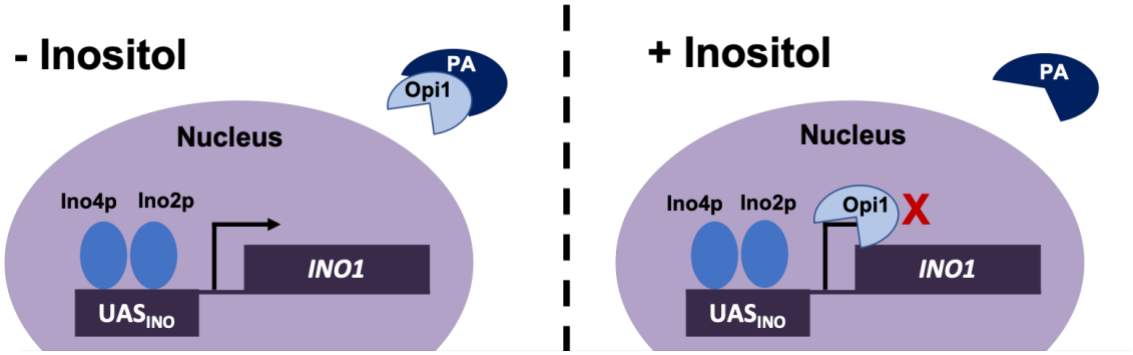

- Inositol
PA
Opi1
Nucleus
Ino4p
Ino2p
INO1
UAS$_{INO}$
+ Inositol
PA
Nucleus
Ino4p
Ino2p
Opi1
X
INO1
UAS$_{INO}$

Figure 1.1: The regulation of INO1 transcription is mediated by the repressor protein, Opi1.

In the absence of inositol, the Opi1 repressor is maintained in the ER by phosphatidic acid (PA). This allows for the expression of *INO1* (left). In the presence of inositol, the cell does not need to expend cellular energy to synthesize inositol, therefore Opi1 dissociates from PA and translocates to the nucleus, where it prevents *INO1* transcription (right).

1.4 Unfolded Protein Response

ER stress is the imbalance between the protein folding capacity and the protein folding load in the ER. The cellular response to this imbalance is the activation of the UPR, which restores ER homeostasis through three primarily adaptive mechanisms: 1) attenuation of protein translation to reduce the protein load entering the ER, 2) transcriptional activation of UPR genes to increase protein folding capacity, and 3) stimulation of membrane lipid synthesis to expand ER volume[2,4,26]. If ER stress is prolonged, and ER homeostasis cannot be re-established, then a fourth mechanism, cell death, is elicited[2,26,48].

In mammals, perturbations in ER homeostasis are sensed and transduced to the cytoplasm and nucleus via three ER-resident sensors: inositol requiring enzyme 1 (Ire1), activating transcription factor 6 (ATF6), and pancreatic ER eIF2α kinase (PERK; Fig. 1. 2A)[1,4,26,49]. These three sensors share similar structures in that they all harbor luminal, transmembrane, and cytoplasmic domains. In the absence of ER stress the ER chaperone, binding immunoglobulin protein (BiP), binds to the luminal domain of the three sensors, thereby maintaining their inactive states[50–52]. In response to an accumulation of misfolded proteins during ER stress, BiP dissociates from the sensors in order to bind misfolded proteins, and thereby activates and initiates UPR signaling. Of the three sensors, Ire1, is the most conserved branch of the UPR. Following dissociation from BiP, Ire1 oligomerizes, allowing for transautophosphorylation of adjacent kinase domains, and stimulation of cytosolic endoribonuclease activity[1,4,45,53]. Once activated, Ire1 excises an intron from the mRNA of its only known substrate, X-box binding protein 1 (XBP1), thereby generating an active transcription factor. The spliced variant of XBP1 then translocates to the nucleus where it binds to an unfolded protein response element (UPRE) in the promoter sequence of a number of UPR-target genes, thereby regulating their expression. Genes that are regulated by XBP1 include those that mediate ER protein folding, quality control, ERAD, and membrane expansion[54,55]. In addition to the selective cleavage of XBP1 mRNA, Ire1 also alleviates ER stress by reducing protein synthesis through regulated Ire1-dependent decay (RIDD), wherein it degrades a subset of ER-localized mRNA[25]. Further examination of proteins that bind UPR promoter elements led

to the identification of the second protein folding sensor, ATF6. While ATF6 is normally an ER-resident protein, under conditions of ER stress, it is trafficked to the Golgi apparatus where it is cleaved into an active transcription factor[56]. This transcription factor then translocates to the nucleus where it increases the expression of ER-resident molecular chaperones and folding enzymes such as BiP, calreticulin, and protein disulfide isomerase [57]. Finally, the third protein folding sensor, PERK, exerts its function by phosphorylating a component of the translation initiation complex, eukaryotic initiation factor 2 (eIF2α). When eiF2α is phosphorylated, it prevents the downstream formation of the ternary initiation complex eIF2-GTP-tRNA^{MET}i, and thereby leads to global attenuation of protein translation[58–60].

Unlike mammals, the sole ER stress sensor in *S. cerevisiae* is Ire1; however, it functions in a manner similar to Ire1 in metazoans in terms of its cytosolic endonuclease activity[2,3,61](Fig 1.2B). Upon induction of ER stress, the BiP homologue, Kar2, dissociates from the luminal domain of Ire1, allowing it to oligomerize, transautophosphorylate, and activate its cytosolic RNase activity[2–4]. Ire1 then splices *HAC1* mRNA to generate a functional variant of the transcript, which upon translation functions as a transcription factor to upregulate genes involved in ER quality control machinery, ribosome biogenesis, and ERAD components[2,3]. Previous literature has also suggested a role for Hac1 in mediating membrane expansion and lipid biogenesis[62,63]. Taken together, the yeast model of the UPR provides a simplified but representative model through which ER stress signaling may be investigated.

Interestingly, unlike *S. cerevisiae, Schizosaccharomyces pombe*, lack both HAC1/XBP1 orthologs and a UPR-dependent transcriptional program[64]. Instead, under conditions of ER stress, *S. pombe* relies exclusively on two means of Ire1-dependent post transcriptional regulation: 1) RIDD, and 2) processing of Bip1 mRNA within its 3'UTR[64]. The processing of Bip1 mRNA stabilizes Bip1 and ensures that it is present at an increased steady state concentration, without increasing transcription[64]. As such, *S. pombe* corrects the protein folding imbalance by decreasing the protein folding load in the ER. Given that the RIDD function of Ire1 in *S. pombe* is conserved in higher

eukaryotes[64], exploring how it impacts yeast tolerance to ER stressors could be useful in understanding the pathways that mediate protein folding homeostasis.

Although the UPR is well characterized, what remains unclear is how it integrates with other signaling pathways under conditions of ER stress. Interestingly, a reciprocal connection has been identified between the cell wall integrity (CWI) and ER stress pathways[65–67].

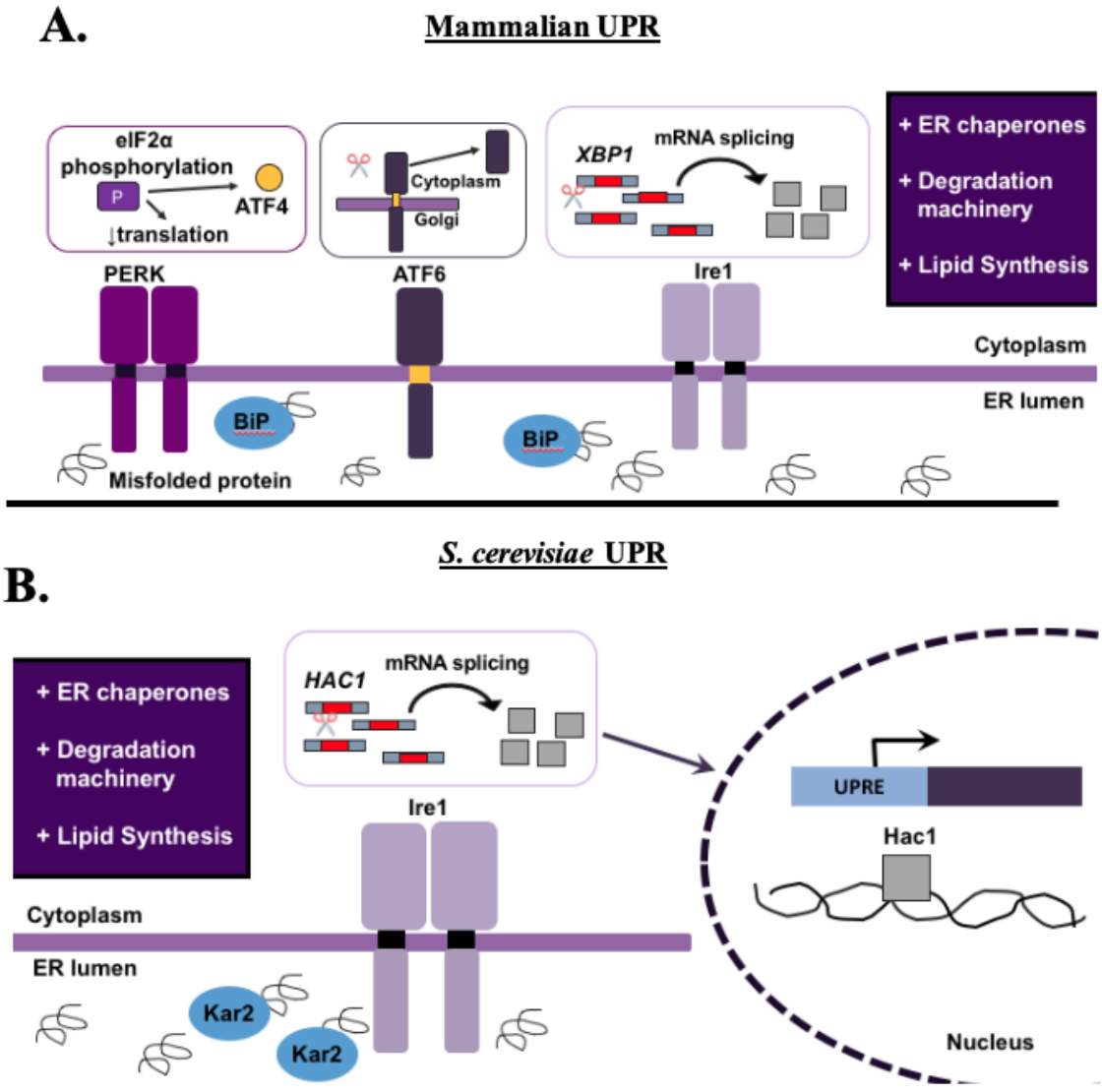

A.
Mammalian UPR
eIF2α
phosphorylation
P
ATF4
↓translation
Cytoplasm
Golgi
XBP1
mRNA splicing
+ ER chaperones
+ Degradation machinery
+ Lipid Synthesis
PERK
ATF6
Ire1
Cytoplasm
ER lumen
BiP
BiP
Misfolded protein
B.
S. cerevisiae UPR
+ ER chaperones
+ Degradation machinery
+ Lipid Synthesis
HAC1
mRNA splicing
Ire1
Cytoplasm
ER lumen
Kar2
Kar2
UPRE
Hac1
Nucleus

Figure 1.2: Representative schematic of the unfolded protein response (UPR).

(A) The mammalian UPR consists of three protein folding sensors: PERK, ATF6, and Ire1. (**B)** *S. cerevisiae* UPR. Ire1 is highly conserved from yeast to mammals.

1.5 Convergence of the UPR and CWI Pathways

Within an external environment, fungi face numerous environmental stressors such as low nutrient availability and changes in pH and temperature[12,68]. As such, the fungal cell wall acts as the first line of defense, providing a rigid cellular boundary to withstand internal turgor pressure and extracellular stresses. Proper cell wall architecture requires three major components: β 1-3-glucan, chitin, and mannoproteins– all of which come together to form a large macromolecular complex [69,70]. In response to environmental stress, the coordinated synthesis of cell wall components occurs through the cell wall integrity (CWI) pathway, which plays an essential role in maintaining cell wall homeostasis[12,71,72]. While the main components of the CWI pathway are conserved in most species of fungi, the foundational understanding of the CWI pathway stems from studies in *S. cerevisiae* [71,73–78]. In *S. cerevisiae*, under conditions of cell wall stress, the CWI pathway responds through a signaling cascade that links cell-surface sensors, Wsc1, Mid2, and Mtl1, to a series of intracellular signaling molecules including the Rho1 GTPase, which binds and activates Pkc1, which in turn activates the MAPK signaling cascade, including Bck1, Mkk1/2, and Mpk1/Slt2[73,74,76] (Fig. 1.3). Phosphorylated Slt2 then translocates to the nucleus where it regulates the expression of cell-wall genes through two distinct pathways: 1) the Rlm1 transcription factor; or 2) the Swi4/6 complex (Fig.1.3). Slt2 activation of the Rlm1 transcription factor allows for the regulation of a number of genes involved in cell wall homeostasis including GPI proteins and chitin synthases[79]. Similarly, Slt2 activation of the Swi4/6 complex not only allows for the transcription of cell-wall related genes like β 1-3-glucan synthases Fks1 and Fks2, but also mediates cell-cycle related genes [80].

Defects in the CWI pathway leads to cell lysis when yeast are exposed to environmental conditions that impair cell wall stability such as high temperature [81] or disruptions in cell wall synthesis [82]. More specifically, loss of function of any component downstream of Pkc1 leads to cell lysis at elevated growth temperatures; however, this growth defect is osmoremedial, with the addition of 1M sorbitol, consistent with a defect in cell wall biogenesis [83,84]. Furthermore, mutants in the CWI pathway are more sensitive to cell wall antagonists such as Calcofluor white [85,86], Congo red [87], and caffeine [88].

Interestingly, the CWI pathway and secretory pathway are reciprocally affected under conditions of environmental stress[12,89,90]. In this regard, defects in CWI cause increased sensitivity to ER stress and impairments in secretory pathway homeostasis disrupt cell wall composition. Surprisingly, deletions in genes involved in the CWI pathway, particularly *BCK1* and *SLT2,* cause extreme sensitivity to the ER stressors, tunicamycin and DTT[66]. This suggests that to compensate for cell wall defects during cell wall stress, the CWI pathway may upregulate a number of cell wall proteins, thereby increasing the protein flux through the ER, and contributing to ER stress. Conversely, ER homeostasis is required for proper cell wall biogenesis and for mediating resistance to the cell wall-targeting drug, caspofungin[67]. Fungal mutants lacking *HAC1* or *IRE1* exhibit increased sensitivity to the cell wall antagonists, Calcofluor white and Congo red[91]. This suggests that ER stress may compromise the fidelity of cell wall proteins, impair biogenesis of cell wall constituents, and consequently activate the CWI pathway. Taken together, the coordination of a number of pathways may be responsible for mediating sensitivity to environmental stressors.

It is also important to note that both cell wall biogenesis and protein folding in the ER are highly energetically demanding processes and, as such, low nutrient status is a potent trigger of the UPR[40]. Thus, the interconnection between metabolic regulation and the UPR is a crucial area of study, one that has thus far been inadequately addressed. Accumulating evidence suggests that the cellular metabolism mediating AMPK signaling cascade and its subsequent regulation of crucial proteins acetyl-CoA carboxylase and mTOR, may cooperate with the UPR to mediate cell viability under conditions of ER stress[40,42,49]; however, the mechanisms behind this crosstalk remain to be elucidated.

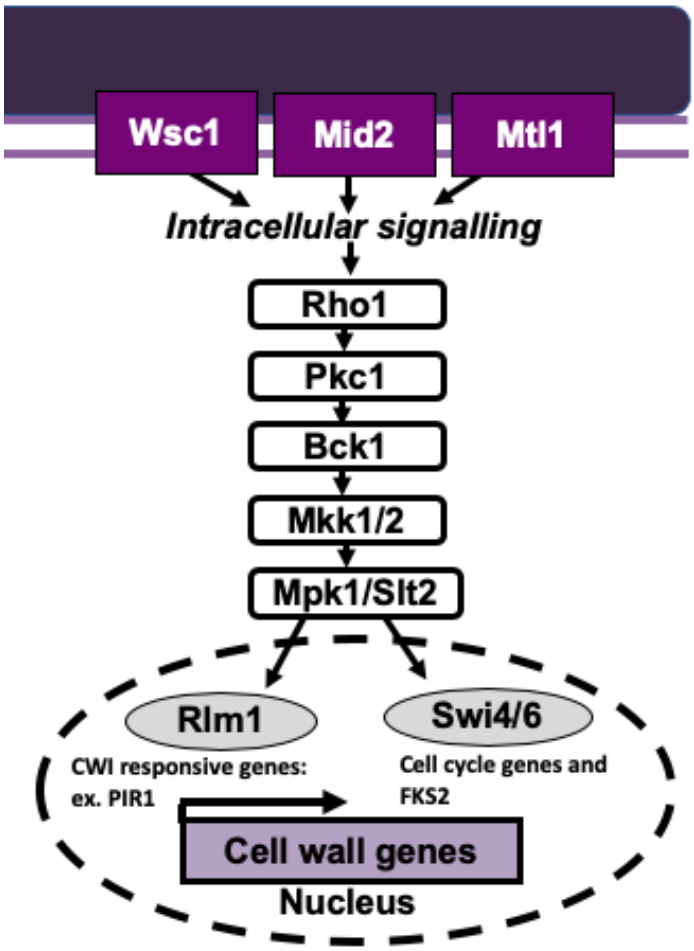

Wsc1
Mid2
Mtl1
Intracellular signalling
Rho1
Pkc1
Bck1
Mkk1/2
Mpk1/Slt2
Rlm1
Swi4/6
CWI responsive genes:
ex. PIR1
Cell cycle genes and
FKS2
Cell wall genes
Nucleus

Figure 1.3: Representative schematic of the cell wall integrity (CWI) pathway in *S. cerevisiae*

In yeast cells, the CWI signaling cascade mediates the transcription of cell wall genes by transducing signals from outer membrane sensors to intracellular effector proteins.

1.6 ER homeostasis and TORC1 Signaling

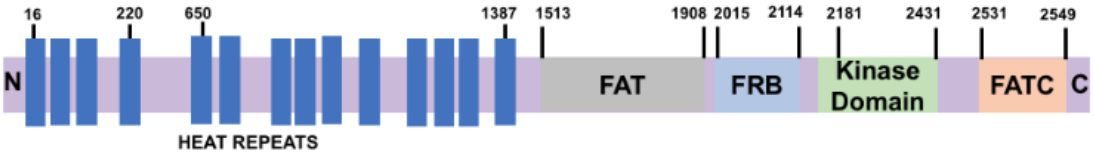
The target of rapamycin (TOR) is an evolutionarily conserved serine/threonine kinase that functions at the core of signaling networks involved in cell growth, metabolism, and nutrient and hormone sensing[92,93]. These signaling networks mediate anabolism and catabolism by coordinating a number of cellular and metabolic processes such as transcription, protein translation, ribosome biogenesis, and cellular architecture[94–97]. *TOR* kinase genes were originally identified by mutations that conferred resistance to the growth inhibitory properties of the drug, rapamycin, in budding yeast, *Saccharomyces cerevisiae* [92]. Unlike yeast which possess two *TOR* genes (TOR1 and TOR2), higher eukaryotes contain only one *TOR* gene (mTOR). Nevertheless, the functional domains within these proteins are highly conserved (Fig. 1.4A). *TOR* genes encode relatively large (~280kDa) proteins that have a conserved C-terminal phosphatidylinositol kinase (PIK) homology domain, an FKBP12-rapamycin binding (FRB) domain, about 20 tandemly repeated HEAT motifs to mediate protein-protein interactions, and FAT and FATC domains which serve as important protein-protein regulators and catalytic activity mediators, respectively[97,98]. In all eukaryotes, these TOR kinases are the central component of two distinct complexes: TOR complex 1 (TORC1) and TOR complex 2 (TORC2), of which only TORC1 is rapamycin sensitive[95].

In particular, TORC1 in yeast has a size of ~2MDa and consists of four main proteins: Kog1, Lst8, Tco89 and either TOR1 or TOR2 (Fig. 1.4B)[99,100]. Localization studies demonstrate that TORC1 is localized to the yeast vacuole and that changes in localization are not necessary for TORC1 signaling [101,102]. A major breakthrough in the field came from the discovery that rapamycin treatment alters cell physiology in a manner similar to nutrient starvation, such that treatment with rapamycin resulted in a decrease in protein synthesis, induction of apoptosis, and entrance into a quiescent G0 state[92]. This observation was the first indication that TORC1 plays an important role in mediating cell growth in response to cell nutrient status. In general, TORC1 is responsible for promoting ribosome biogenesis, cell proliferation, and protein anabolism. In addition to mediating anabolic processes, TORC1 also promotes cell growth by inhibiting a number of stress response pathways[94,103,104]. To date, the best characterized substrate of TORC1 in yeast is

Sch9, the yeast homolog for the AGC kinase, S6K, and monitoring the phosphorylation status of this protein is an indicator of TORC1 activity[101]. Additionally, the phosphorylation of RPS6, a downstream target of Sch9, is regulated in a TORC1-dependent manner and serves as a valid readout for TORC1 activity *in vivo*[105,106]. Recent reports indicate that under conditions of cell stress, Sch9 and RPS6 phosphorylation is dramatically reduced; however, it is unclear how these stress signals are transduced to TORC1[103,105,106].

Interestingly cells treated with rapamycin activate the CWI pathway, suggesting that the TORC1 signaling pathway not only impinges upon the CWI pathway, but also negatively regulates the pathway[107,108]. Therefore, while initially thought to be distinct pathways, recent research points to a functional interaction between the UPR, TORC1, and CWI signaling pathways[104,107–109]. Nevertheless, the manner in which the CWI, UPR, and TOR signaling pathways interact remains to be elucidated. Given that these signaling pathways are all essential for facilitating fungal pathogenesis, understanding the manner in which these pathways act in parallel to mediate ER homeostasis may allow us to uncover novel targets for antifungal drugs.

A.


16
220
650
1387
1513
1908
2015
2114
2181
2431
2531
2549
N
FAT
FRB
Kinase Domain
FATC
C
HEAT REPEATS

B.

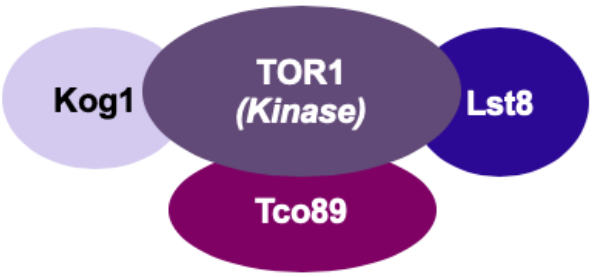

Kog1
TOR1
(Kinase)
Lst8
Tco89

Figure 1.4: Representative schematic of the TOR1 Kinase and TORC1 complex.

A) Conserved structure of TOR kinases**.** The functional domains of TOR include tandem HEAT repeats, the FAT domain, the FRB-rapamycin binding domain, the PIK homologous kinase domain, and the FATC domain. **B)** Representative schematic of the TORC1 complex. The central component of the complex is the TOR1 kinase.

1.7 Cellular Pathways mediating Pathogenic Fungal Infections

The prevalence of pathogenic fungal infections, coupled with the emergence of new fungal pathogens, has rapidly brought these diseases to the forefront of global health problems [13]. Nearly 2 billion people are afflicted by fungal infections worldwide, resulting in 1.5 million deaths annually [110,111]. While most individuals will suffer from generally treatable superficial fungal infections, of particular concern are the millions of people worldwide that will contract life-threating invasive infections – diseases with a mortality rate which exceeds 50%, even with the availability of antifungal treatments [111,112]. Despite the wide-spread prevalence of these diseases, the study of fungal infections is greatly lacking when compared to other infectious diseases. As such, there is a pressing need for research in this field to explore the cellular pathways mediating infection in order to facilitate the development of novel therapeutics.

The most widespread group of superficial mycoses are fungal infections of the skin and nails, affecting nearly 25% of the world's population[112]. These infections are primarily caused by the fungal agents, dermatophytes, and give rise to conditions such as athlete's foot, ringworm of the scalp, and infection of the nails [112]. Other superficial fungal infections include mucosal infections of the oral and genital tract, such as oropharyngeal- or vulvovaginal candidiasis respectively, commonly known as thrush[113,114]. The vast majority of these superficial mucosal infections are caused by several species of *Candida*, the second most prevalent fungal species worldwide [113–115]. Individuals who are particularly susceptible to mucosal fungal infections include transplant patients, individuals diagnosed with leukemia, and patients who have undergone radiotherapy[111]. While superficial fungal infections are relatively well managed with antifungals, recurrent infections show decreased sensitivity to antifungal compounds[110,114] and bring forth the problem of fungal resistance.

While the incidence of invasive fungal infections (IFI) is significantly lower than that of superficial fungal infections, IFI pose a much greater threat because of their high mortality rates[111,116]. The large majority of deaths caused by invasive fungi are often attributed to opportunistic infections, such that fungal pathogens take advantage of hosts

with a compromised immune system. As such, patients with the greatest risk of contracting these life-threatening infections include those diagnosed with immunosuppressive diseases such as asthma and HIV/AIDs [117–119] or those treated with immunosuppressive medical interventions such as chemotherapy and radiation [120]. As a whole, the aetiological agents responsible for more than 90% of IFI-related deaths fall largely within four genera of fungi: *Cryptococcus, Candida, Aspergillus,* and *Pneumocytis* [111,121]. While antifungal treatments have advanced over the last decade, patient outcomes have not substantially improved [122]. These shortcomings are largely attributed to the evolutionary similarity between fungi and humans, which limits the scope of drug development against fungal specific targets. As such, there is a pressing need to understand the unique cellular mechanisms that govern fungal viability.

Since the cell wall is essential for fungal survival and its composition is unique to the fungal organism, this structure acts as an ideal target for antifungal drugs[123]. Notably, echinocandins represent the first class of antifungal drugs that specifically target the fungal cell wall[124,125]. In particular, the echinocandin caspofungin acts as a fungicide by noncompetitively inhibiting the β 1-3-glucan synthases, Fks1 and Fks2, thereby blocking cell wall synthesis[126]. Genome-wide microarray analysis of yeast cells treated with caspofungin revealed that treatment with this drug rapidly and specifically triggers induction of CWI related genes[70]. This observation was confirmed by Northern blot analysis, which demonstrated that caspofungin induced Slt2 phosphorylation[70]. Moreover, cells with deletions in crucial CWI genes, Slt2, Bck1, Pkc1, and Fks1/2, were all hypersensitive to caspofungin, suggesting that integrity of CWI pathway is required for tolerance to caspofungin[70]. Notably, caspofungin also induced the expression of the chitin synthase, Chs1p[70]. This is in line with previous studies, which reported that cells lacking Fks1/2 have a compensatory mechanism induced, resulting in higher chitin and mannoprotein content [127–129]. Given the dual regulation of Fks1/2 by the calcineurin pathway[128,130] (Fig. 1.5), it was also interesting to note that caspofungin caused repression of the calcineurin gene, *CNA1* [70,131]. Furthermore, pathogenic fungi rely heavily on the secretory pathway to govern tolerance to antifungal drugs[132,133], mediate cell wall homeostasis[134,135], and express virulence[136]. Therefore, exploring the connection between

the CWI pathway, UPR, and nutrient sensing TOR pathway, is integral for understanding the pathways that mediate fungal pathogenesis.

Given that *S. cerevisiae* is evolutionarily related to a number of pathogenic fungi, and in particular to the *Candida* species[137], most genes from *S. cerevisiae* are highly conserved in pathogenic fungal strains. This conserved homology makes *S. cerevisiae* an ideal model system to identify signal transduction and metabolic pathways required for fungal survival in the host environment. Among the shared genomic features includes similar mechanisms for cell wall homeostasis[138–140] and activation of stress responses[141]. Therefore, *S. cerevisiae* is a powerful tool to analyze the integration of cellular pathways that mediate fungal viability.

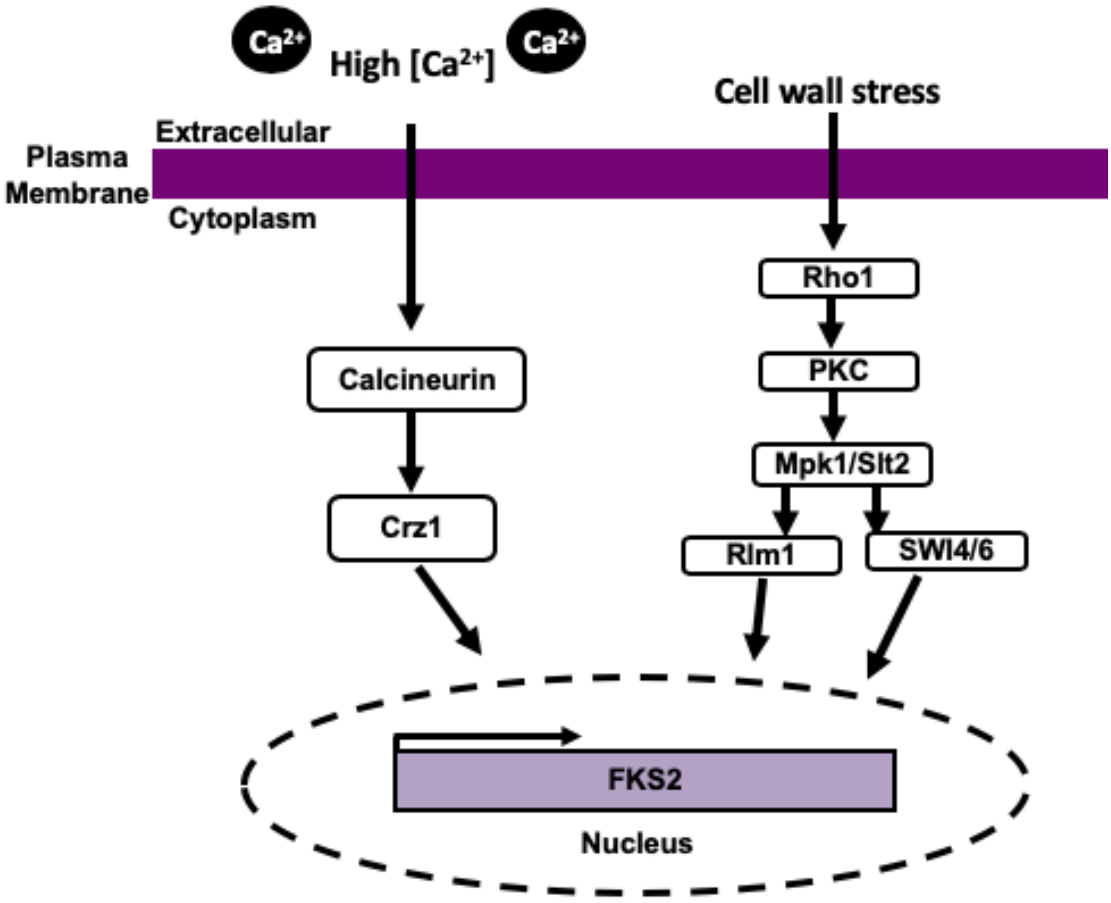

Ca2+
High [Ca2+]
Ca2+
Cell wall stress
Extracellular
Plasma Membrane
Cytoplasm
Rho1
Calcineurin
PKC
Mpk1/Slt2
Crz1
Rlm1
SWI4/6
FKS2
Nucleus

Figure 1.5: Ca^{2+}/Calcineurin signaling and CWI converge to mediate *FKS2* gene expression.

The calcium/calcineurin signaling pathway and the CWI pathway dually regulate expression of *FKS2* gene expression under conditions of cell wall or high calcium stress.

1.8 Hypothesis

My central hypothesis is that attenuation of TORC1 signaling during ER stress is required for β 1-3 glucan synthase expression and adaptation to proteotoxic stress. Overall, the goal of this research is to better understand the cross-talk between the TOR signaling pathway, cell wall integrity pathways, and the UPR, and how the interplay between these pathways mediates ER stress sensitivity (Fig. 1.6).

Both TORC1[92] and UPR[3,142] signaling were initially characterized in yeast and are conserved in higher eukaryotes. Therefore, I rationalized that employing the model organism, *Saccharomyces cerevisiae*, would be a powerful tool to investigate the interplay between the UPR and other signaling networks. Among the advantages of employing yeast is that it is a genetically and biochemically tractable model organism that allows for rapid and extensive genetic manipulation. Taken together, yeast will provide an excellent platform to analyze the integration of cellular pathways during ER stress.

1.9 Objectives

To study my hypothesis, I have three main objectives:

Objective 1: Determine the effect of TORC1 signaling on ER stress sensitivity

Objective 2: Examine how hyperactivation of TORC1 signaling sensitizes cells to ER stressors

Objective 3: Determine the role of TORC1 signaling in mediating cell wall integrity during ER stress

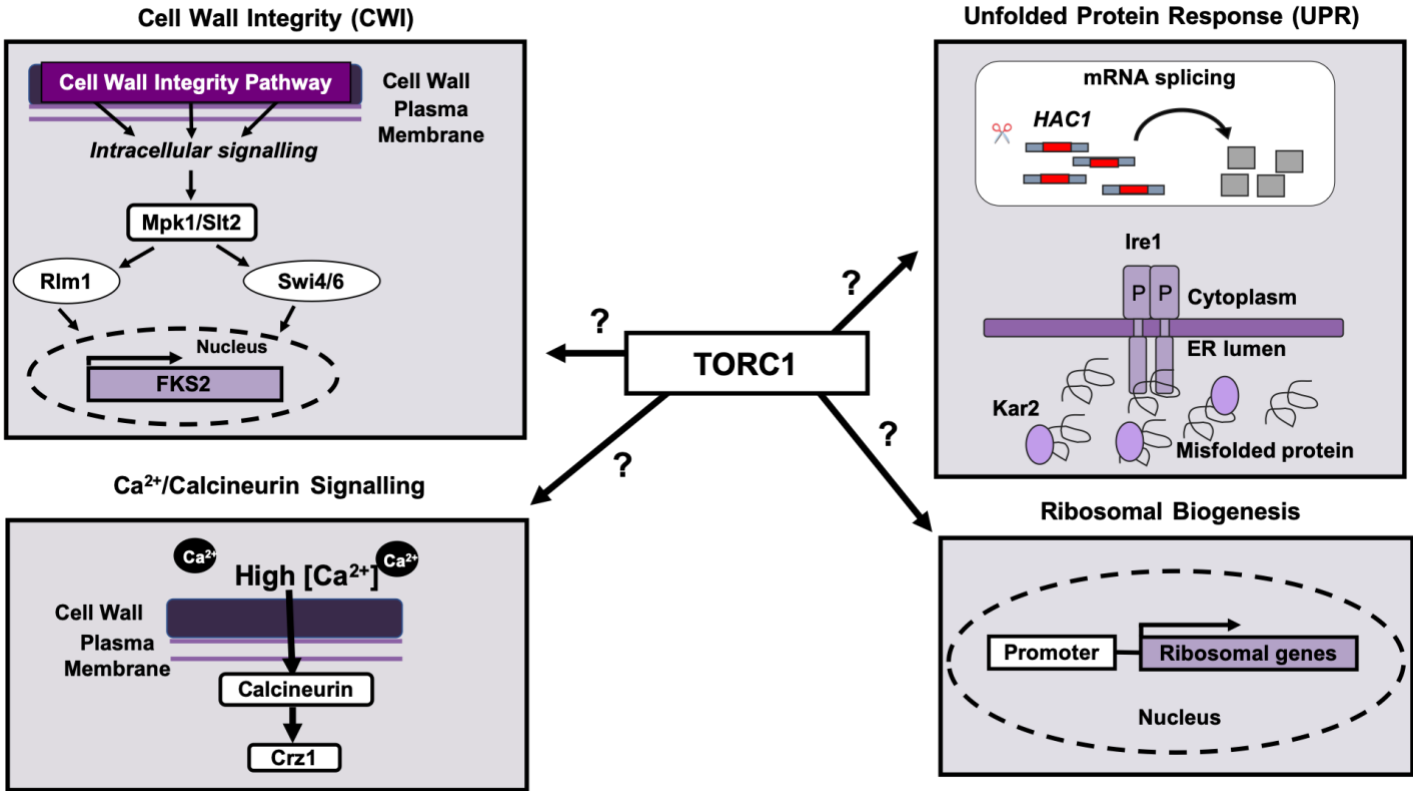

Cell Wall Integrity (CWI)
Cell Wall Integrity Pathway
Cell Wall
Plasma
Membrane
Intracellular signalling
Mpk1/Slt2
Rlm1
Swi4/6
Nucleus
FKS2
Unfolded Protein Response (UPR)
mRNA splicing
HAC1
Ire1
P
P
Cytoplasm
ER lumen
Kar2
Misfolded protein
?
?
?
?
TORC1
Ca2+/Calcineurin Signalling
Ca2+
High [Ca2+]
Ca2+
Cell Wall
Plasma
Membrane
Calcineurin
Crz1
Ribosomal Biogenesis
Promoter
Ribosomal genes
Nucleus

Figure 1.6: TORC1 signaling during ER stress.

It is unclear how TORC1 signaling acts in parallel with the UPR to mediate ER stress sensitivity. We seek to investigate whether TORC1 signaling has a role in mediating cell wall architecture, ribosomal biogenesis, and UPR integrity during ER stress.

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Chapter 2

2 Hyperactive TORC1 Sensitizes Yeast Cells to Endoplasmic Reticulum Stress by Compromising Cell Wall Integrity

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2.1 Introduction

The ability of cells to respond to detrimental stresses, such as an aberrant accumulation of toxic misfolded proteins, dictates cell fate under both normal and pathological conditions. Loss of secretory protein homeostasis due to pharmacological, genetic, or environmental perturbations activates a plethora of adaptive responses to help cells overcome the stress [1,2]. In yeast, the ER resident protein Ire1 detects changes in the ER misfolded protein and activates a transcriptional response termed the unfolded protein response (UPR; [3–7]. Upon induction of ER stress, the ER chaperone, Kar2, dissociates from the luminal domain of Ire1, allowing it to oligomerize, trans-autophosphorylate, and subsequently activate its cytosolic RNase activity [4,5,8–10]. Ire1 then splices *HAC1* mRNA to generate a functional variant of the transcript, which upon translation functions as a transcription factor to upregulate genes involved in ER quality control machinery and ribosome biogenesis [5,8]. Cellular adaptation to ER stress is not only dependent on the amplitude of the UPR signal, but also on the selective expression of UPR target genes capable of overcoming a particular stress condition [11]. Interestingly, Pincus *et al*. (2014) show that *S. cerevisiae* amplify the UPR with time delayed Ras/PKA signaling, indicating that the response to ER stress is not limited to the UPR [12]. Moreover, induction of ER stress activates transcription of genes associated with other types of stress responses [2]. Therefore, elucidating how the UPR integrates with other signaling pathways under conditions of ER stress is essential to understand how proteostasis is mediated in the cell.

Given that protein folding in the ER is a highly energetically demanding process, low nutrient status is a potent trigger of the UPR [13]. Therefore, the interconnection between metabolic regulation and the UPR is a crucial area of study, one that has thus far been inadequately addressed. Accumulating evidence suggests that the cellular metabolism mediating AMPK signaling cascade and its subsequent regulation of crucial proteins acetyl-CoA carboxylase and TOR, may cooperate with the UPR to mediate cell viability under conditions of ER stress [13–15]; however, the mechanisms behind this crosstalk remain to be elucidated. In yeast, TORC1 inhibition with rapamycin protects yeast cells from ER stress-induced vacuolar fragmentation and promotes antifungal synergism [16]. In addition, pharmacological induction of ER stress triggers autophagy, a process negatively

regulated by TORC1 [17]. It therefore appears that TOR signaling is an important determinant of the yeast ER stress response.

In *S. cerevisiae*, TOR kinases are evolutionarily conserved serine/threonine kinases that function at the core of signaling networks involved in cell growth, metabolism, and nutrient and hormone sensing [18,19]. These TOR kinases are the central component of two distinct complexes: TOR complex 1 (TORC1) and TOR complex 2 (TORC2), of which only TORC1 is rapamycin sensitive [20]. In particular, the TORC1 signaling network mediates anabolism and catabolism by coordinating cellular and metabolic processes such as transcription, protein translation, ribosome biogenesis, and cellular architecture [20–23]. In addition to mediating anabolic processes, TORC1 promotes cell growth by inhibiting a number of stress response pathways [21,24,25]. Nevertheless, the manner in which the secretory and TORC1 signaling pathway act in parallel, under conditions of ER stress, remains to be elucidated.

To study the effect of TORC1 signaling on protein folding homeostasis, we employed a hyperactive variant of the TOR1 kinase (*TOR1*^{L2134M}) [15] and assessed yeast sensitivity to ER stress. We elucidate a novel interplay between proteostasis and TORC1 signaling and show that attenuation of TORC1 signaling is required for adaptation to ER stress. On the other hand, constitutive activation of TORC1 confers increased sensitivity to ER stressors, including the antifungal caspofungin, by compromising cell wall architecture. Our study, therefore, expands the role of ER homeostasis beyond the UPR and defines how TORC1 signaling contributes to the ER stress response.

2.2 Materials and Methods

2.2.1 Yeast Strains and Methods

The *Saccharomyces cerevisiae* strains and plasmids used in this study are listed in Tables 2.1 and 2.2, respectively. All yeast strains are derivatives of BY4742. The TS161 (*TOR1*) and TS184 (*TOR1*^{L2134M}) strains were kind gifts from Dr. Maeda[24]. BY4742 or derivatives were thawed from frozen stocks and grown on YPD (yeast extract peptone

dextrose) or selective SC (synthetic complete) media for 2 days at 30°C before being transferred to liquid cultures. All experiments were carried out using either SC media containing 2% wv^{-1} glucose supplemented with 100x inositol or YPD media. Cultures were grown at 30°C with constant agitation or on selective agar plates.

Table 2.1: Yeast Strains

Strains	Genotype	Reference
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	26, 27
TS161 Wild-type TOR	*MATα ura3-52*	24
TS184 Mutant TOR	*MATα ura3-52 TOR1L2134M*	24
BY4742 i*re1Δ*	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 IRE1::KAN*	Deletion collection

Table 2.2: Plasmids

Plasmids	Number	Vector Backbone	Resistance	Reference
pPM47 (UPR-RFP CEN/ARS URA3)	Addgene plasmid # 20132	pRS316	URA	28
pAMS366 (*4X CDRE-lacZ* URA3)	_	pAMS366	URA	29
pRS316 BCK1-20	–	pRS316	URA	30
pRS416 GPD	ATCC 87360	pRS416	URA	31

2.2.2 Spotting and Liquid Growth Assays

Cell growth was assessed by both spot assay and liquid culture as previously described by Duennwald (2013). Briefly, spotting assays were performed with yeast cells that were cultured overnight in selective media with 2% glucose as the sole carbon source. Cells were then diluted to equivalent concentrations of OD_{600} 0.2 and were spotted in 4 sequential five-fold dilutions. Equal spotting was controlled by simultaneously spotting cells using a multi-channel ultra-high-performance pipette (VWR International). Cells were grown on selective plates at 30°C for 2 days and imaged using a Geldoc system (Bio-RAD). For liquid cultures cells were diluted to OD_{600} 0.15 and incubated at 30°C. OD_{600} was measured every 15 mins using a BioscreenC plate reader (Growth curves

USA) for 24 h. Growth curves were generated and the area under the curve was calculated for biological replicates. Statistical significance was determined using a two-tailed student T-test and GraphPad (Prism).

2.2.3 Yeast Transformation

Yeast transformations were performed using the lithium acetate transformation protocol as previously described[32]. Briefly, 1 mL of OD_{600} = 1, overnight cultures were pelleted at 3000 xg for 1 min. Cells were aspirated and washed with 1.5 mL sterile 0.1 M LiAc in TE buffer. Cells were then pelleted and resuspended in 285 μL sterile 50% PEG 4000 in 0.1M LiAc, 2.5 μL plasmid, and 10 μL boiled salmon sperm DNA, and incubated at 30°C for 45 mins. After that, 43 μL of sterile DMSO was added and cells were heat shocked for 15 min at 42°C before being plated on amino acid selection plates.

2.2.4 Drugs

Stock solutions of tunicamycin (5 μg mL^{-1} in DMSO; Amresco), calcofluor white (30 mg mL^{-1} in H_2O; Sigma Aldrich), rapamycin (1 mg ml^{-1} in DMSO; Fisher Bioreagents), sorbitol (3 M in H_2O; Fisher Bioreagents), and fluorescent brightener 28 (Calcofluor white stain; 25μM; Sigma Aldrich) were used at the indicated concentrations.

2.2.5 Stress Condition Experiments

In all the experiments, yeast cultures were grown to log phase (OD_{600} ~0.3) before being exposed to different stress conditions. Endoplasmic reticulum stress was achieved by adding 0.5 μg mL^{-1}, 1.0 μg mL^{-1}, or 2.5 μg mL^{-1} tunicamycin (Amresco) or by inositol withdrawal. For inositol depletion experiments, cells were washed twice in SC media (YNB-Inositol; Sunrise Science) and then resuspended into pre-warmed SC media lacking inositol. Cell wall stress was achieved by adding 5-20 μg mL^{-1} calcofluor white. Sorbitol rescue assays were facilitated by adding 1 M sorbitol to the media.

2.2.6 Quantitative RT-PCR

RNA extraction was performed using the MasterPure Yeast RNA Purification Kit (Epicentre). cDNA was synthesized using the RevertAid H Minus First Strand cDNA

Synthesis Kit (Thermoscientific). The cDNA preparations were used as templates for amplification using SsoAdvanced^{Tm} Universal SYBR ® Green Supermix (Bio-Rad). The primers used are listed in Table 2.3. The relative expression levels were calculated using the comparative Ct method with U3 as a reference gene.

Table 2.3: Primers

Gene	Forward Primer	Reverse Primer
U3	CCCAGAGTGAGAAACCGAAA	AGGATGGGTCAAGATCATCG
KAR2	CCGGTGAAGAAGGTGTCGAA	CATGGCTCTTTCACCCTCGT
RPL30	ATCATTGCCGCTAACACTCC	CCGACAGCAGTACCCAATTC
INO1	TCGACGTACAAGGACAACGA	GGCCACTAAAGTGGAGCCAT
HAC1	ACGACGCTTTTGTTGCTTCT	TCTTCGGTTGAAGTAGCACAC
PRM5	GACATAAGGAAACCCGCAAA	CCAGCATGTGCTCGAGATAA
FKS2	CTGAGCGCCGTATTTCATTT	CGGGTGTAATTGCTTCAGGT
FKS1	TTTGGTTCCAATTGGGTGTT	CCGCAAACACTTCGAACATA
FIT1	GTGAACGTGCTCCTGTCTCA	GTTCACCCTCACCAGTCCAT
FIT2	GACACCGCTGACCCTATCAT	GATGATTCGACGGCTTGAGT
FIT3	TATCACTGCCACCAAGAACG	AATTCAGCGGTGCTAGAGGA

2.2.7 Fluorescence Microscopy

TOR1 and *TOR1*^{L2134M} cells expressing a UPR-mcherry fluorescent reporter were grown to mid-log phase before being treated with 2.5 μg mL^{-1} tunicamycin (Amresco) or inositol withdrawal for 3 h. Cells were diluted 10X, transferred to a 96 well plate, and imaged at room temperature. Fluorescence microscopy was performed using the Cytation 5 Cell Imaging Multi-Mode Reader (BioTek); the 20X objective lens and Texas Red Filter cube (586 647^{-1} nm) were used. Images were analyzed using ImageJ software (https://imagej.nih.gov/ij/). Violin plots presented in Figure 2.2D were generated using the PlotsOfData software [33].

2.2.8 HAC1 Splicing Assay

Cells were cultured to mid-log phase before being treated with either 1.0 μg/mL tunicamycin (Amresco) or inositol withdrawal for 2 h. RNA extraction was performed using the MasterPure Yeast RNA Purification Kit (Epicentre). cDNA was synthesized from the extracted RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit

(Thermoscientific). The cDNA preparations were then used as templates for RT-PCR with *HAC1* primers (listed in Table 4). The resulting reaction product was separated by electrophoresis on an agarose gel and bands were visualized using a Geldoc system (Bio-Rad).

2.2.9 β-galactosidase Assay

TOR1 and *TOR1*^{L2134M} yeast strains transformed with plasmids carrying the *CDRE-LacZ* reporter were assayed as previously described [34]. Briefly, cells were grown to log phase in selective SC media, harvested by centrifugation, then cultured in SC media containing the indicated concentrations of stressors or $CaCl_2$. After incubation at 30°C for 2 h, cells were harvested by centrifugation and resuspended in lacZ buffer. To measure β-galactosidase activity, 50 μL cell lysate was mixed with 950 μL lacZ buffer containing 2.7 μL β-mercaptoethanol, 1 drop 0.1% SDS, 2 drops $CHCl_3$ and incubated at 30°C for 15 min. The reaction was started by adding 100 μL ONPG (4 mg mL^{-1}) and incubated at 30°C till the colour changed to yellow. The reaction was stopped by adding 300 μL of 1 M Na_2CO_3. β-galactosidase activity was determined at 420 nm absorbance using a plate reader, normalizing data to cell density.

2.2.10 Protein Extraction and Western Blot

Cells were lysed using alkaline lysis with 0.1 M NaOH [35] and proteins were extracted into 4x Laemmli sample buffer containing 100 mM DTT. Protein samples were separated using SDS-PAGE (BioRad Mini-PROTEAN TGX Pre-Cast gels, 4-15%) and transferred to nitrocellulose membranes using the BioRad Trans-Blot® Turbo™ RTA Transfer Kit. Membranes were blocked with 5% fat free milk for 30 mins, before probing with P-S6 Ribosomal Protein S235 236^{-1} Rabbit Ab (Cell Signaling Technology) or anti-PGK1 (Invitrogen) overnight at 4°C. Membranes were then incubated with the Alexa Fluor 488 goat anti-rabbit for 1 hr. Membranes were imaged using a BioRad infrared imager (BioRad).

2.2.11 Calcofluor White Stain Microscopy and Flow Cytometry

TOR1 and *TOR1*^{L2134M} cells were grown in triplicate to mid-log phase in YPD media, before being treated with Fluorescent Brightener 28 (Sigma-Adlrich) to a final concentration of 25 μM. Cells were grown for 20 min at 30°C with continuous shaking before they were pelleted and washed in SC media. Cells were diluted 10x in growth media and plated in Lab-Tek (Thermo Inc.) imaging chambers and processed for fluorescence microscopy. Images were acquired using a Zeiss AxioVert A1 wide filed fluorescence microscopy equipped with a 63X NA 1.4 Plan Apopchromat objective, 359 nm excitation 461 nm^{-1} emission (DAPI) long pass filter and an AxioCam ICm1 R1 CCD camera (Carl Zeiss inc.). Images were analyzed using ImageJ software. For flow cytometric analysis, cells were cultured in appropriate media and processed for flow cytometry using a BD Bioscience FACS Celesta flow cytometer equipped with a 405 nm Violet laser. Data was analyzed using the BD FACS Diva Software. All conditions were performed in triplicate, 20 000 cells were analyzed, and mean fluorescence intensities were calculated. No gates were applied.

2.2.12 Microarray Analysis

TOR1 and *TOR1*^{L2134M} yeast cultures were grown to log phase (OD_{600} ~0.3) before being treated with tunicamycin (2.5 μg/mL). RNA was extracted from two independent cultures (n=2) and quality was assessed with Bioanalyzer as previously described [36]. Microarray analysis was conducted with the GeneChip® Yeast Genome 2.0 Array (Affymetrix, Santa Clara, California, USA). Briefly, biotinylated complimentary RNA (cRNA) was prepared from 100 ng of total RNA as per the GeneChip 3' IVT PLUS Reagent Kit manual (ThermoFisher Scientific, Waltham, MA). Data was analyzed using the Transcriptome Analysis Console (TAC) software (Affymetrix) by filtering for genes that showed a two-fold change in expression with a p-value of 0.05 using sacCer3 as a reference genome. Gene lists were created using the gene ontology term finder on the *Saccharomyces* genome database (https://www.yeastgenome.org/). All microarray data were submitted to the GEO database as series GSE129200.

2.3 Results and Discussion

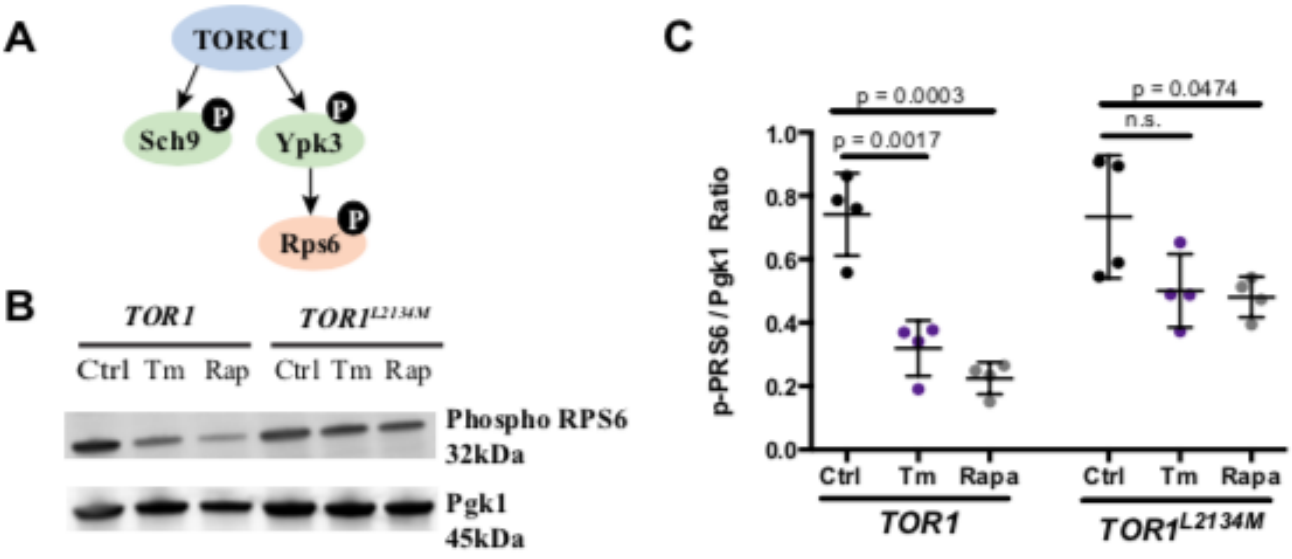
2.3.1 Hyperactive *TOR1*^{L2134M} sensitizes cells to ER stress

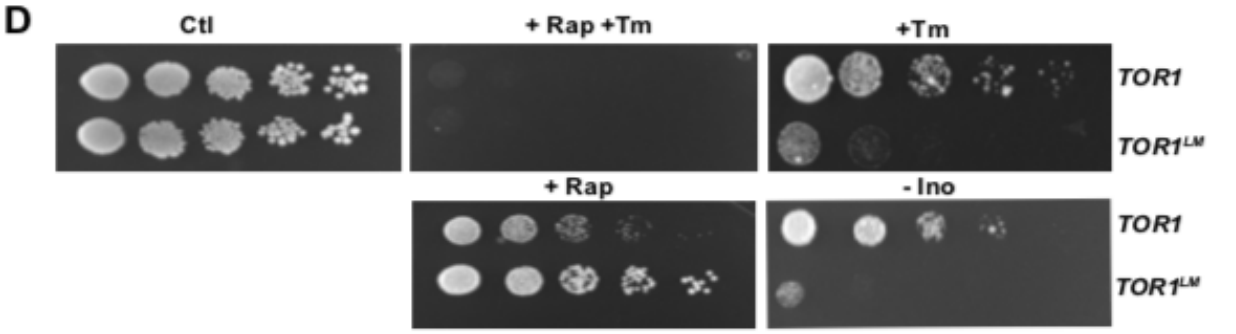
Previous studies show that the TOR pathway links nutrient status to cell growth and ribosome biogenesis, under conditions of protein misfolding stress [37–39]. However, it remains unclear to what extent modulation of TORC1 signaling is required for adaptation to ER stress. Thus, we sought to investigate the effects of TORC1 signaling on the sensitivity to ER stress.

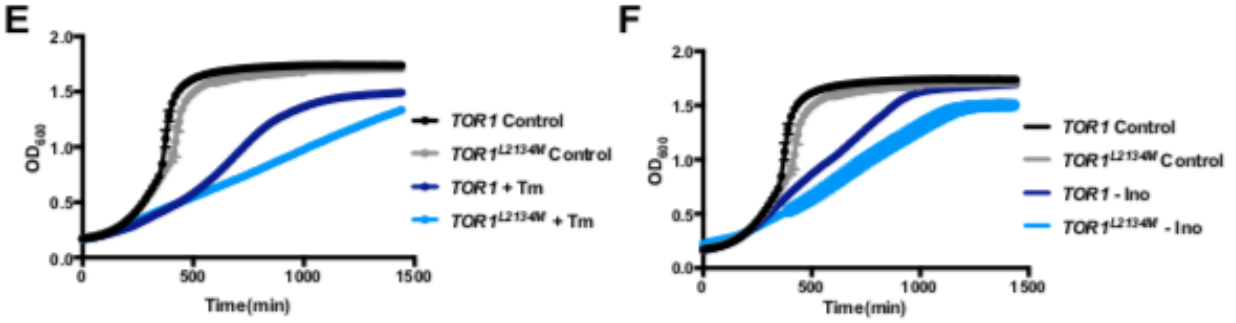
The phosphorylation of the ribosomal protein, RPS6, is regulated in a TORC1-dependent manner and serves as a valid readout for TORC1 activity *in vivo* [40,41]. Previous reports indicate that under conditions of oxidative- and proteotoxic stress, RPS6 phosphorylation is dramatically reduced [42,43]. Therefore, we sought to investigate whether ER stress downregulates RPS6 phosphorylation in cells with hyperactive TORC1 signaling (Fig. 2.1A). As such, cells expressing either WT *TOR1* or hyperactive *TOR1*^{L2134M} were treated with the canonical ER stress inducer, tunicamycin (Tm; Fig. 2.1B). Tm is a potent inducer of the UPR as it inhibits N-glycosylation of proteins, prevents proper protein folding, and thereby causes an accumulation of misfolded proteins in the ER [44]. While the addition of Tm (2.5 ug/mL) significantly decreased RPS6 phosphorylation in cells expressing WT *TOR1*, there was no significant difference in cells expressing hyperactive *TOR1*^{L2134M} (Fig. 2.1B-C). Rapamycin, an inhibitor of TORC1, was used as a positive control, for Sch9 downregulation. Combined with previous studies showing that phosphorylation of Sch9, another TORC1 effector, is decreased during Tm treatment [45], our results suggest that TORC1 deactivation plays an important role in ER stress tolerance. As such, we then sought to determine how impacting proper TORC1 signaling affects the cell's response to ER stressors.

First, we assessed cell growth in the presence of both Tm and the TORC1 inhibitor, rapamycin (Fig. 2.1D). We found that rapamycin treatment exacerbates the growth defect caused by Tm-induced ER stress (Fig. 2.1D). Similarly, cells expressing a rapamycin-resistant hyperactive *TOR1*^{L2134M} [24] displayed an increased growth defect upon Tm stress (Fig. 2.1D). To investigate the effects of hyperactive *TOR1* on a more physiologically

relevant ER stressor, cells were exposed to conditions of inositol withdrawal. While it is unclear how exactly inositol deprivation triggers UPR activation, some studies have postulated that it triggers the UPR by either changing the lipid composition of the ER membrane [46–48] or by impairing membrane trafficking [49,50]. In contrast to cells expressing WT *TOR1*, cells expressing the hyperactive allele were inositol auxotrophs (Fig. 2.1D). Increased ER stress sensitivity of $TOR1^{L2134M}$ was confirmed using liquid growth assays (Fig. 2,1E-F). As expected, compared to cells expressing WT *TOR1*, cells expressing hyperactive $TOR1^{L2134M}$ had a significant growth defect following treatment with Tm (Fig. 1E) or inositol withdrawal (Fig. 2.1F). Taken together, our results indicate that defective TORC1 signaling increases sensitivity to canonical ER stressors. Both phenotypes can be linked to a defective response to ER stress.







Control	AUC
TOR1	129.7 ± 1.5
$TOR1^{L2134M}$	124.0 ± 1.5

+ Tm	AUC
TOR1	87.4 ± 0.9
$TOR1^{L2134M}$	70.8 ± 1.5*

- Ino	AUC
TOR1	106.2 ± 0.9
$TOR1^{L2134M}$	88.8 ± 4.2*

Figure 2.1: Cells expressing hyperactive *TOR1*^{L2134M} are more sensitive to ER stress

(A) Representative schematic of the downstream targets of TORC1 kinase activity. **(B)** Western blot analysis of RPS6 phosphorylation following treatment with tunicamycin (Tm; 2.5 μg/mL) or rapamycin (Rap; 200 ng/mL). Pgk1 was used as a loading control. **(C)** Quantification of (B). Sch9 phosphorylation is not attenuated in hyperactive *TOR1*^{L2134M} cells following treatment with tunicamycin (n=4; ± SD). **(D)** Cell growth of WT *TOR1* and *TOR1*^{L2134M} cells was assessed by serial dilutions on YPD plates supplemented with rapamycin (Rap; 10 ng/mL), tunicamycin (Tm; 1.0 μg/mL), both Rap and Tm, or SC plates supplemented without inositol (+/- Inositol). Cells expressing hyperactive *TOR1*^{L2134M} were more resistant to rapamycin treatment and more sensitive to tunicamycin stress and inositol withdrawal. **(E)** Liquid growth assays of yeast cells expressing WT *TOR1* and *TOR1*^{L2134M} were used to further assess sensitivity to tunicamycin stress (Tm; 1.0 μg/mL) and **(F)** inositol withdrawal (-Ino). Data is quantified as area under the curve (AUC; *$p < 0.01$; mean ± SD; n=3).

2.3.2 Cells expressing hyperactive *TOR1*^{L2134M} have a functional UPR

Having shown that cells expressing hyperactive *TOR1*^{L2134M} are more sensitive to ER stress, we next sought to examine whether this increased sensitivity was due to defects in the ability to activate the UPR. As previously described, under conditions of ER stress, the ER protein folding sensor, Ire1, splices HAC1 mRNA to produce an active transcription factor [4]. We therefore assessed the ability of Ire1 to splice *HAC1* mRNA using RT-PCR (Fig. 2.2A-B). Surprisingly, inositol withdrawal induced *HAC1* splicing in both WT *TOR1* and hyperactive *TOR1*^{L2134M} mutants (Fig. 2.2A, arrow). Additionally, after 1 hr of treatment with Tm, cells expressing hyperactive *TOR1*^{L2134M} spliced *HAC1* mRNA, and this response was still evident after 2 hrs of induction, as indicated by a smaller fragment in the agarose gel (Fig. 2.2B, arrow). As a whole, these results indicate that increased ER sensitivity of cells expressing hyperactive *TOR1*^{L2134M} is not due to impaired functionality of the UPR.

Spliced *HAC1* mRNA is translated into an active transcription factor, which then translocates to the nucleus where it binds to unfolded protein response element (UPRE) sequences in gene promoters[44]. In response to ER stress, Hac1 alone activates over 400 UPR target genes, including ER chaperones, genes that mediate membrane expansion, and genes involved in ribosome biogenesis [1,51,52]. As such, increased sensitivity to ER stress may be due to an inability to transcriptionally activate the UPR. We tested this possibility by transforming a UPRE-mcherry fluorescent reporter [28] into cells expressing *TOR1* and *TOR1*^{L2134M} and assessing UPR activation with fluorescence microscopy (Fig. 2.2C-D). Surprisingly, there was no significant difference between cells expressing *TOR1* and hyperactive *TOR1*^{L2134M} in their ability to activate the UPR under conditions of Tm stress and inositol withdrawal. Additionally, we quantitatively assessed the mRNA levels of the yeast resident chaperone and canonical UPR target gene, *KAR2,* using qRT-PCR (Fig. 2.3A). In line with our previous data, hyperactive *TOR1*^{L2134M} was able to increase the expression of *KAR2*, following treatment with Tm and inositol withdrawal. Taken together, these results suggest that the increased sensitivity of cells expressing *TOR1*^{L2134M} to ER stress is unlikely to be due to impaired UPR activation.

Additionally, actively dividing yeast allocate up to 85% of their transcriptional activity to ribosome biogenesis [53]; however, under conditions of ER stress, there is a downregulation in the expression of ribosome genes in order to increase the expression of UPR target genes [54,55]. As such, we employed qRT-PCR to assess the expression of *RPL30*, a gene involved in ribosome biogenesis (Fig. 2.3B). Cells expressing hyperactive $TOR1^{L2134M}$ significantly downregulated expression of *RPL30* (Fig. 2.3B). This is probably due to the fact that multiple pathways regulate ribosome biogenesis. For example, PKA deactivation during ER stress is also responsible for repressing transcription of ribosomal protein genes [12]. Furthermore, depleting inositol triggers the ER sensor, Ire1, which induces transcription of the inositol biosynthetic gene, *INO1* [8,56]. Therefore, we investigated whether the inositol auxotrophy of cells expressing $TOR1^{L2134M}$ was due to the inability to synthesize *INO1*. Cells expressing *TOR1* and $TOR1^{L2134M}$ were treated with inositol withdrawal and qRT-PCR was conducted to assess the expression of *INO1* and *RPL30* (Fig. 2.3C-D). Interestingly, hyperactive $TOR1^{L2134M}$ impaired the transcription of *INO1* (Fig. 2.3C) but did not impair ribosome biogenesis (Fig. 2.3D). Taken together, these results suggest that under conditions of ER stress, cells expressing hyperactive $TOR1^{L2134M}$ are defective in regulating *INO1* transcription.

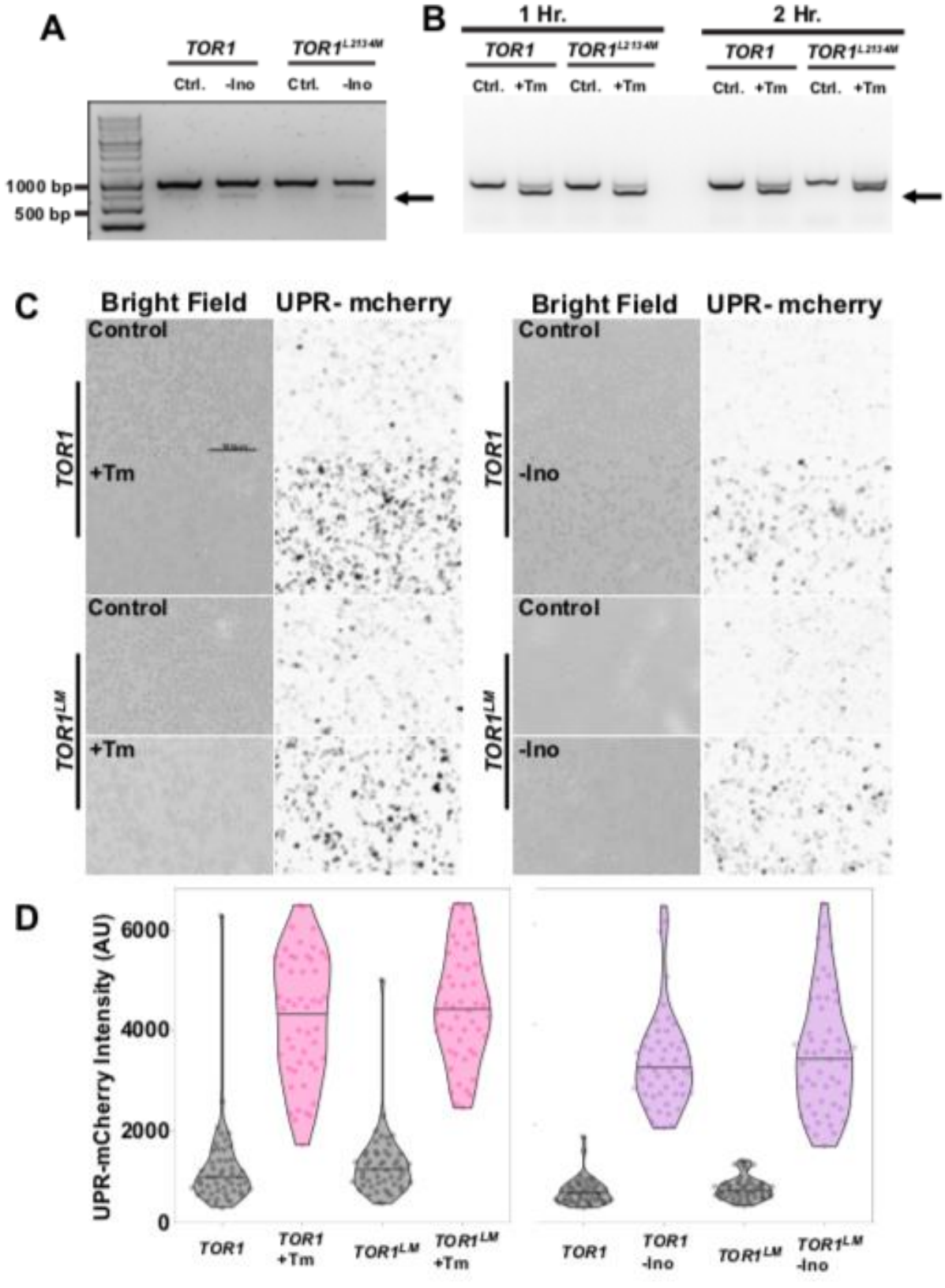

A
TOR1
TOR1L213A4M
Ctrl.
-Ino
Ctrl.
-Ino
1000 bp
500 bp
B
1 Hr.
2 Hr.
TOR1
TOR1L213A4M
Ctrl.
+Tm
Ctrl.
+Tm
TOR1
TOR1L213A4M
Ctrl.
+Tm
Ctrl.
+Tm
C
Bright Field
UPR- mcherry
Bright Field
UPR- mcherry
Control
TOR1
+Tm
Control
TOR1LM
+Tm
Control
TOR1
-Ino
Control
TOR1LM
-Ino
D
UPR-mCherry Intensity (AU)
6000
4000
2000
0
TOR1
TOR1 +Tm
TOR1LM
TOR1LM +Tm
TOR1
TOR1 -Ino
TOR1LM
TOR1LM -Ino

Figure 2.2: The UPR is not impaired in yeast cells expressing hyperactive *TOR1*^{L2134M}

(A) Treatment with ER stressors induces HAC1 mRNA splicing. WT *TOR1* and hyperactive *TOR1*^{L2134M} mutants were either untreated (Ctrl.), treated with inositol withdrawal (-Ino), or **(B)** treated with tunicamycin (Tm; 1.0 μg/mL) for up to 2 hrs. RT-PCR was conducted using *HAC1* primers. Arrows indicate Ire1 mediated HAC1 splicing. (**C)** Representative fluorescence microscopy images of WT *TOR1* and *TOR1*^{L2134M} cells expressing UPR-mcherry fluorescent reporters, following treatment with tunicamycin (Tm; 1.0 μg/mL) and inositol withdrawal (-Ino) for 2 hours. **(D)** Quantification of (C). Hyperactive *TOR1*^{L2134M} promotes expression of UPR-induced genes in conditions of tunicamycin stress and inositol withdrawal (n=50; *$p < 0.0001$).

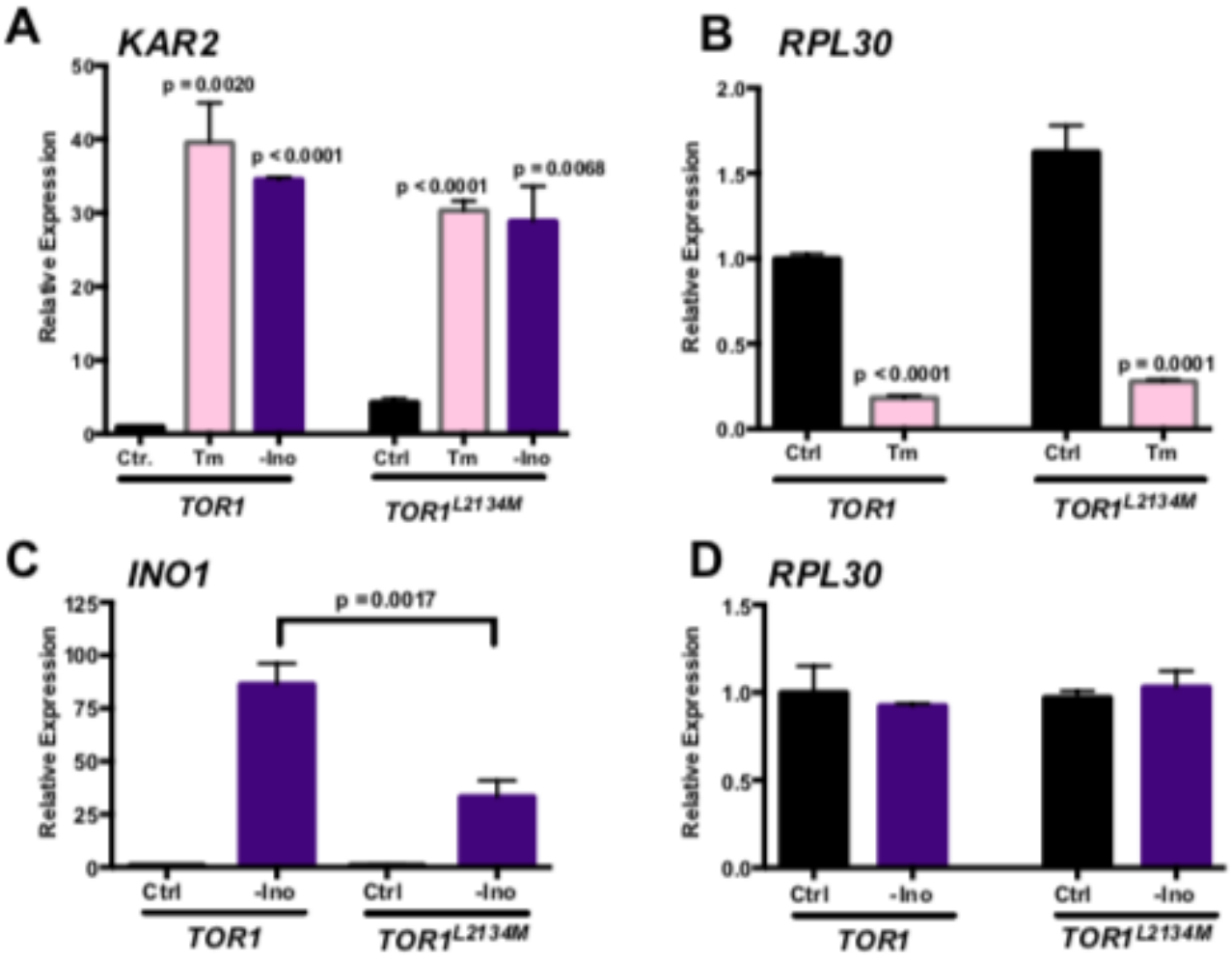

A
KAR2
Relative Expression
p = 0.0020
p < 0.0001
p < 0.0001
p = 0.0068
Ctr.
Tm
-Ino
Ctrl
Tm
-Ino
TOR1
TOR1L2134M
B
RPL30
Relative Expression
p < 0.0001
p = 0.0001
Ctrl
Tm
Ctrl
Tm
TOR1
TOR1L2134M
C
INO1
Relative Expression
p = 0.0017
Ctrl
-Ino
Ctrl
-Ino
TOR1
TOR1L2134M
D
RPL30
Relative Expression
Ctrl
-Ino
Ctrl
-Ino
TOR1
TOR1L2134M

Figure 2.3: Hyperactive *TOR1*^{L2134M} can transcriptionally activate the UPR, but has impaired inositol synthesis

(A) Representative fluorescence microscopy images of WT *TOR1* and *TOR1*^{L2134M} cells expressing UPR-mcherry fluorescent reporters, following treatment with tunicamycin (Tm; 1.0 μg/mL) for 2 hours. **(B)** Quantification of (A). Both WT *TOR1* and hyperactive *TOR1*^{L2134M} have significantly increased UPR-mcherry fluorescence following treatment with tunicamycin (n=50; *$p<0.05$). **(C)** Fluorescence microscopy of WT *TOR1* and *TOR1*^{L2134M} cells expressing UPR-mcherry fluorescent reporters, following treatment with inositol withdrawal (- ino). **(D)** Quantification of (C). Hyperactive *TOR1*^{L2134M} promotes expression of UPR-induced genes in conditions of inositol withdrawal (n=50; *$p<0.05$).

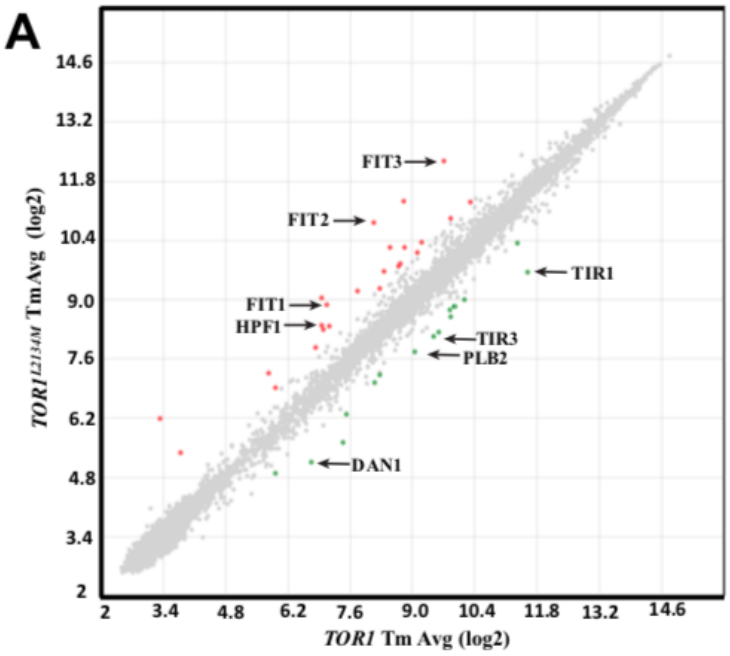
2.3.3 Defects in cell wall integrity underlie *TOR1*^{L2134M} sensitivity to ER stress

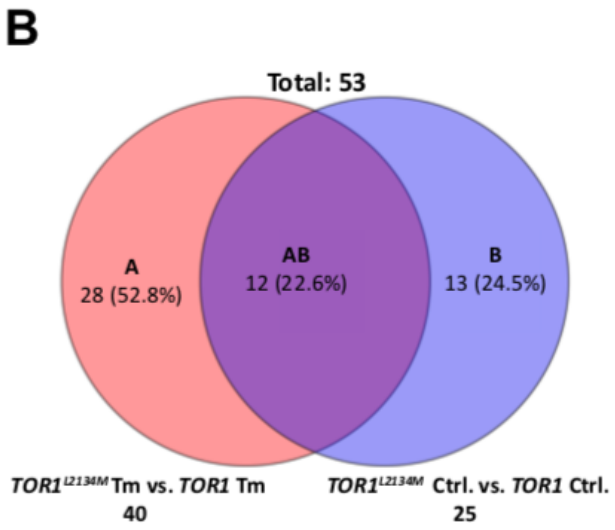
Despite having a functional UPR, our studies show that cells expressing hyperactive *TOR1*^{L2134M} have increased sensitivity to canonical ER stressors. Therefore, to assess how ER stress alters the transcriptome in hyperactive *TOR1*^{L2134M} mutants, we treated two independent cultures of WT *TOR1* and hyperactive *TOR1*^{L2134M} cells with Tm and used microarray analysis to uncover genes that were differentially expressed in hyperactive *TOR1*^{L2134M} cells (Fig. 2.4A-D). Data was analyzed by filtering for genes that showed a two-fold change in expression with a p value < 0.05. The transcripts of the genes that were differentially downregulated (Fig. 2.4C) and upregulated (Fig. 2.4D) were categorized based on their cellular components using the yeast SGD GO term finder. Interestingly, among the genes that were upregulated, a large majority encoded proteins that localized to the cell periphery and plasma membrane (Fig. 2.4D). Of note, genes encoding three cell wall incorporated mannoproteins, *FIT1, FIT2,* and *FIT3* were upregulated in hyperactive *TOR1*^{L2134M} cells (Fig. 2.4D). Fit proteins are involved in iron uptake [57]. Validation with qRT-PCR revealed that hyperactive *TOR1*^{L2134M} cells had significantly higher steady-state levels of *FIT1, FIT2,* and *FIT3,* compared to cells expressing WT *TOR1* (Fig. 2.4E-G). Interestingly, *FIT* genes are also upregulated in cells carrying deletions in genes encoding the phosphatases *PTC1* and *PTC6* that displayed compromised TORC1 signaling [58]. Additionally, the expression of both *FIT2* and *FIT3* was significantly higher compared to WT *TOR1* cells following treatment with Tm (Fig. 2.4F-G). Interestingly, increased mannoprotein levels is observed in cells with compromised cell wall [59]. Taken together, these results suggest that hyperactive *TOR1*^{L2134M} alters the cell wall composition of yeast cells.

ER stress tolerance in yeast depends on the activation of the cell wall integrity pathway, which is, in part, regulated by TORC1 [60–64]. Additionally, cells with defects in cell wall integrity exhibit inositol auxotrophy [65]. As such, we investigated whether the increased sensitivity of cells expressing hyperactive *TOR1*^{L2134M} was due to defects in cell wall integrity. A general approach to assess whether a specific phenotype is due to a cell wall defect is to test the remediating effects of the cell wall stabilizer sorbitol [66]. Interestingly,

supplementing with sorbitol rescued the toxicity caused by Tm stress in hyperactive *TOR1*^{L2134M} mutants (Fig. 2.5A), suggesting that these cells have a defective cell wall. To further examine cell wall composition, cells expressing *TOR1* and *TOR1*^{L2134M} were treated with the cell wall antagonist, calcofluor white (CFW) and liquid growth assays were assessed (Fig. 2.5B). In line with our previous results, cells expressing hyperactive *TOR1*^{L2134M} were significantly more sensitive to CFW than cells expressing WT *TOR1* (Fig. 2.5B). Previous literature indicates that due to increased activation of cell wall stress responses, yeast strains with defects in cell wall integrity have a greater deposition of chitin in their cell wall and become more sensitive to the CFW [67]. Therefore, cells expressing *TOR1* and *TOR1*^{L2134M} were stained with CFW and chitin staining was analyzed using fluorescence microscopy and flow cytometry (Fig. 2.5C). Compared to WT *TOR1* cells, cells expressing hyperactive *TOR1*^{L2134M} appeared more clustered and displayed significantly more chitin content (Fig. 2.5C). Taken together, our data suggests that the increased sensitivity of hyperactive *TOR1*^{L2134M} mutants can be traced back to defects in cell wall integrity.

Consistent with a defect in cell wall biogenesis, loss of function of any kinase downstream of the canonical MAPK cell wall integrity pathway (CWI) results in growth defects at elevated temperatures [68–71]. Therefore, we investigated whether the increased sensitivity of hyperactive *TOR1*^{L2134M} to ER stress could be attributed to defects in the canonical CWI pathway. Surprisingly, compared to WT *TOR1* cells, cells expressing hyperactive *TOR1*^{L2134M} showed no growth defect at elevated temperatures (Fig. 2.5D). To further investigate whether the CWI pathway was impaired, we assessed the effects of constitutive activation of the CWI pathway by transforming a hyperactive *BCK1-20* allele into WT *TOR1* and hyperactive *TOR1*^{L2134M} cells (Fig. 2.5E). Interestingly, *BCK1-20* overexpression equally rescued Tm toxicity in both WT *TOR1* and hyperactive *TOR1*^{L2134M} cells (Fig. 2.5E), with *TOR1*^{L2134M} cells still displaying increased sensitivity compared to wild-type. These results indicate that other regulators of the cell wall composition downstream of Bck1 may be defective in the mutant cells.





Downregulated

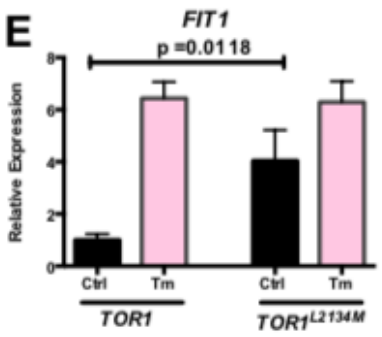
C

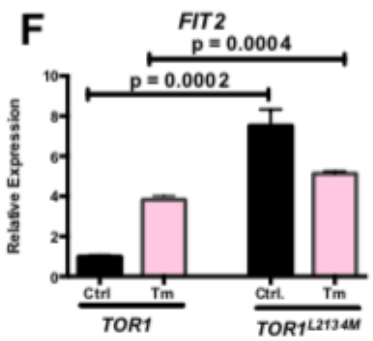
Gene Ontology Term	Genes Annotated to Term
Anchored component of plasma membrane	TIR3, DAN1, TIR1, PLB2, PRM6
Cell Wall	TIR3, PLB2, DAN1, TIR1
Cytoplasm	COS12, APA1, IMD2, HSP26
Nucleus	MAL33, APA1
Cell periphery	HXT4
Endoplasmic Reticulum	TDA4

Upregulated

D

Gene Ontology Term	Genes Annotated to Term
Cell periphery	RSB1, FRE4, SUL1, SIT1, FIT2, FIT1 ARN1, ARN2, FIT3, ENB1, HPF1
Integral component of plasma membrane	ENB1, SUL1, ARN2, ARN1, SIT1
Anchored component of plasma membrane	HPF1, FIT1, FIT2, FIT3
Intrinsic component of plasma membrane	FIT2, FIT1, SIT1, SUL1, RBS1, FRE4 HPF1, ODC2, ENB1, FIT3, ARN2, ARN1
Cytoplasm	ARO10, SNO1, ARG1, ARO9, LSO1 LYS1, ARG3
Mitochondrion	LYS20, ODC2





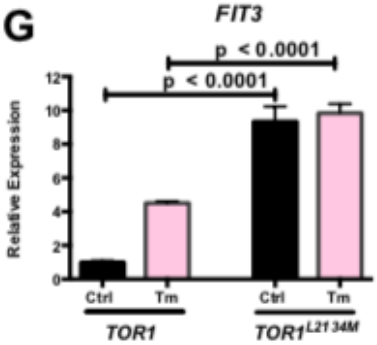
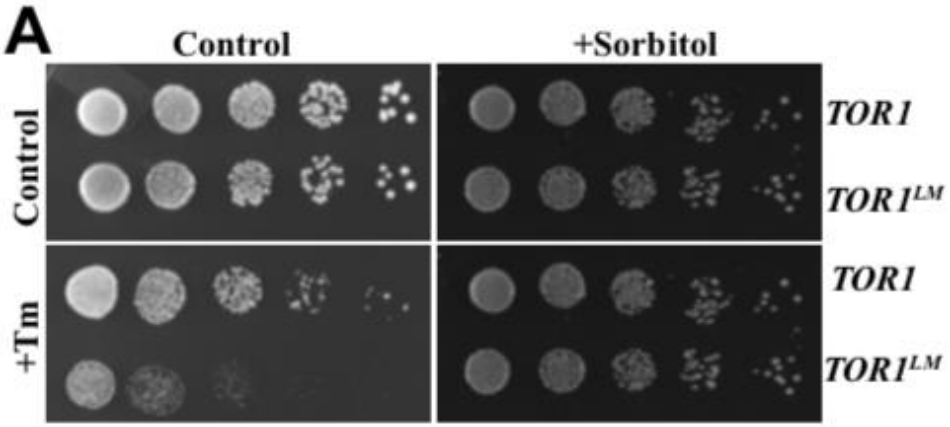
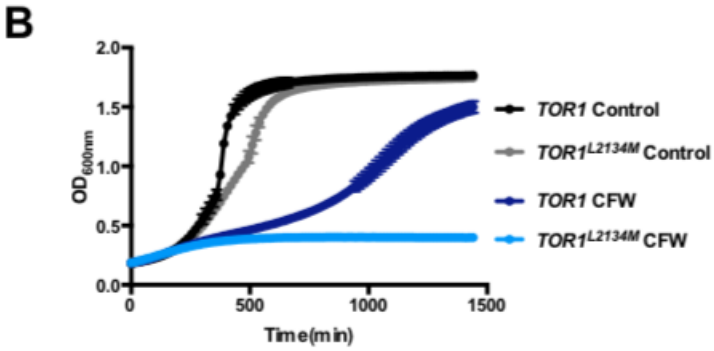


Figure 2.4: ER stress induces a change in the cell wall composition of cells expressing hyperactive *TOR1^{L2134M}*

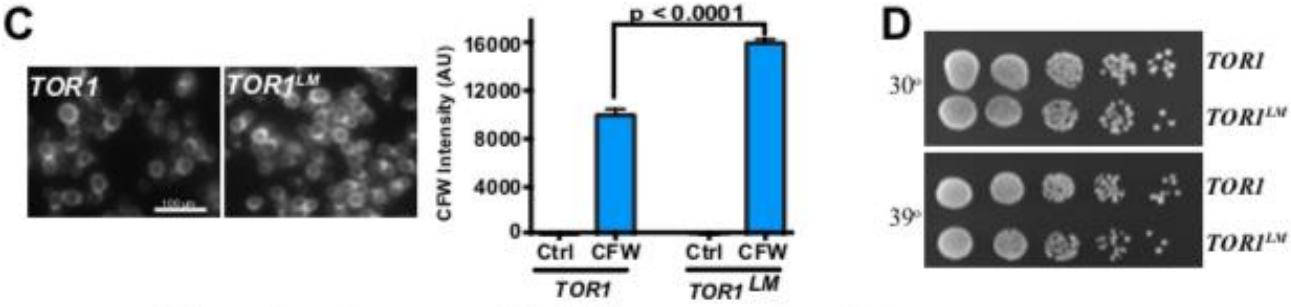
(A) Microarray analysis of genes differentially expressed in yeast cells expressing WT *TOR1* or hyperactive *TOR1^{L2134M}*, following treatment with tunicamycin (Tm; 2.5 μg/mL). Arrows indicate cell wall genes that are differentially expressed in cells expressing hyperactive *TOR1^{L2134M}*. **(B)** Microarray analysis of genes differentially expressed in *TOR1* and *TOR1^{L2134M}* control cells compared to *TOR1* and *TOR1^{L2134M}* cells treated with tunicamycin (Tm; 2.5 μg/mL). **(C)** Genes downregulated two-fold in hyperactive *TOR1^{L2134M}* cells in response to tunicamycin stress (Tm; 2.5 μg/mL). **(D)** Genes upregulated two-fold in hyperactive *TOR1^{L2134M}* cells in response to tunicamycin stress. Gene ontology lists were generated with the gene ontology term finder on the *Saccharomyces* genome database. Numerous cell wall genes are differentially expressed in hyperactive *TOR1^{L2134M}* cells compared to cells expressing WT *TOR1*. **(E)** qRT-PCR was used to validate the microarray analysis and assess expression of mannoprotein genes *FIT1*, **(F)** *FIT2*, and **(G)** *FIT3* following treatment with tunicamycin (Tm; 2.5 μg/mL; n=3; ± SD).





Control	AUC
TOR1	129.9 ± 1.2
$TOR1^{L2134M}$	120.9 ± 0.5

+ Tm	AUC
TOR1	69.8 ± 2.5
$TOR1^{L2134M}$	34.4 ± 0.2*



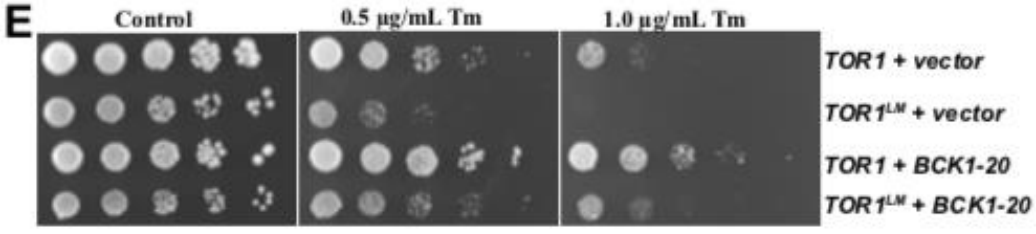


Figure 2.5: Increased sensitivity of hyperactive *TOR1^{L2134M}*, in response to ER stress, is due to defects in cell wall integrity

(A) Cell growth of WT *TOR1* and *TOR1^{L2134M}* cells was assessed by serial dilutions on YPD plates supplemented with various concentrations of tunicamycin (Tm), sorbitol (1 M), or both tunicamycin and sorbitol. Sorbitol rescues tunicamycin toxicity caused by hyperactive *TOR1^{L2134M}*. **(B)** Liquid growth assay of *TOR1* and *TOR1^{L2134M}* cells following treatment with calcofluor white (CFW; 20 μg/mL). Data was quantified by measuring area under the curve (AUC; n=3; *p < 0.001; mean ± SD). **C)** Representative fluorescence microscopy images of cells expressing WT *TOR1* and hyperactive *TOR1^{L2134M}*, following treatment with calcofluor white (CFW; 20 μg/mL). Cells expressing hyperactive *TOR1^{L2134M}* are aggregated and have increased fluorescence, corresponding to an increase in chitin synthesis (Left panel). Flow cytometric analysis of cells treated with calcofluor white (CFW; 2.5 μg/mL). Cells expressing hyperactive *TOR1^{L2134M}* have significantly higher mean fluorescence intensity compared to WT TOR cells (right panel; n = 3; mean ± SD). **(D)** Growth of WT *TOR1* and *TOR1^{L2134M}* cells in response to elevated temperature was assessed by serial dilution on YPD plates. There was no growth defect caused by hyperactive *TOR1^{L2134M}*. **(E)** Cell growth of WT *TOR1* and *TOR1^{L2134M}* transformed with either an empty vector or BCK1-20 was assessed by serial dilution on SC-ura plates supplemented with various concentrations of tunicamycin (Tm).

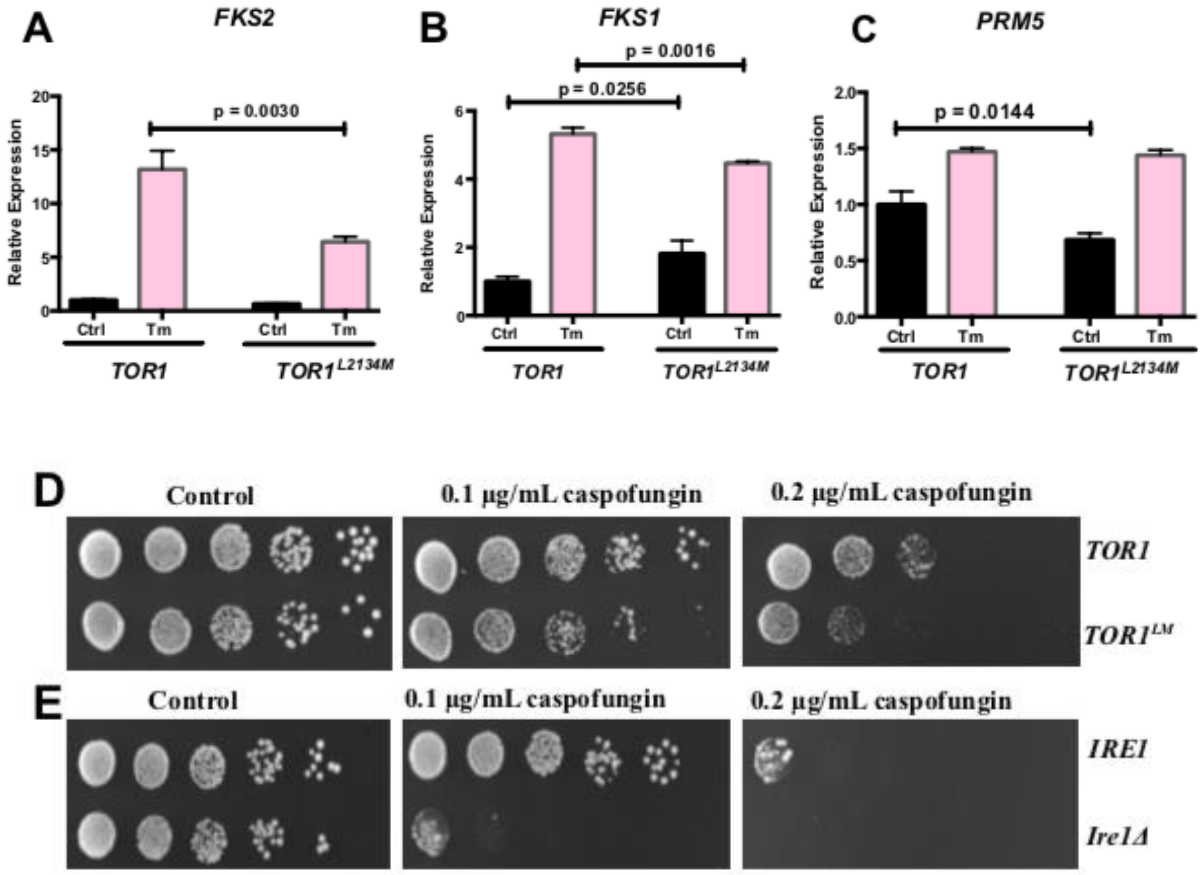
2.3.4 Hyperactive *TOR1*^{L2134M} cells have defects in glucan synthase expression and are more sensitive to the antifungal, caspofungin

Within the host organism, pathogenic fungi face numerous environmental stressors such as low nutrient availability and changes in pH and temperature [72,73]. As such, the fungal cell wall acts as the first line of defense, providing a rigid cellular boundary to withstand internal turgor pressure and extracellular stresses [74]. Proper cell wall architecture requires three major components: β-1-3-glucan, chitin, and mannoproteins– all of which come together to form a large macromolecular complex [74,75]. Our results indicate that cells expressing hyperactive *TOR1*^{L2134M} increase expression of mannoprotein genes as well as chitin aggregation, both of which are phenotypes associated with impaired β-1-3-glucan synthesis [76–78]. To test this possibility, we used qRT-PCR to assess the expression of the β-1-3-glucan synthase genes, *FKS2* and *FKS1* (Fig. 2.6A-B). Interestingly, expression of both *FKS2* (Fig. 2.6A) and *FKS1* (Fig. 2.6B) was significantly decreased in hyperactive *TOR1*^{L2134M} cells, following treatment with Tm. Given that Ca^{2+}/ calcineurin and CWI signaling converge to mediate *FKS1/2* expression [77,79], we differentially assessed the activity of these pathways. There was no evidence that the Ca^{2+}/ calcineurin pathway was impaired in presence of Tm-induced ER stress (Fig. 2.7). Additionally, we examined the activation of Rlm1 – another transcription factor regulating cell wall integrity– by assessing the expression of its downstream target, *PRM5* (Fig. 2.6C). We found that activation of the Rlm1 branch was not impaired in hyperactive *TOR1*^{L2134M} cells (Fig. 2.6C). Taken together, our results support the notion that defects in the cell wall architecture of hyperactive *TOR1*^{L2134M} mutants may be due to dysregulation of other regulators of the cell wall integrity such as the SWI4/6-SBF complex. More comprehensive studies will be required to uncover the complex role of TORC1 in the control of cell wall biogenesis and maintenance.

Given that the cell wall is essential for fungal survival and its composition is unique to the fungal organism, this structure acts as an ideal target for antifungal drugs [80]. Notably, echinocandins represent the first class of antifungal drugs that specifically target the fungal cell wall [81,82]. In particular, the echinocandin caspofungin acts as a fungicide by noncompetitively inhibiting the β-1-3-glucan synthases, Fks1 and Fks2, thereby blocking

cell wall synthesis [83]. Since our results indicate that hyperactive *TOR1*^{L2134M} impairs *FKS2* and *FKS1* synthesis, we investigated whether this defect sensitizes cells to the antifungal, caspofungin (Fig. 2.6D). Indeed, cells expressing hyperactive *TOR1*^{L2134M} exhibited a growth defect as compared to WT *TOR1* cells, and this defect was further exacerbated with increasing concentrations of caspofungin (Fig. 2.6D). To further elucidate the connection between ER stress signaling and sensitivity to antifungal drugs, we examined the growth of *ire1Δ* cells following treatment with caspofungin (Fig. 2.6E). Compared to wild-type strains, *ire1Δ* showed hypersensitivity to caspofungin, suggesting that a functional ER stress response is required for resistance to this antifungal drug (Fig. 2.6E). Similarly, UPR-deficient strains of pathological fungi such as *C. neoformans* and *A. fumigatus* show decreased virulence in animal models [84–87]. Interestingly, deletion of *MDS3* in *Candida albicans* leads to TORC1 hyperactivation resulting in filamentation defects, supporting a negative role for TORC1 hyperactivation in pathogenicity [88]. Conversely, reduced TORC1 signaling in *oma1*Δ strains resulted in attenuated TORC1 signaling and increased virulence in *Candida albicans* [89]. Thus, the amplitude of TORC1 signaling emerges as an important determinant of the capacity of *C. albicans* cells to withstand stress such as oxidative stress [90] and perhaps ER stress, thus impacting its virulence and pathogenicity.

While initially described as distinct pathways, our research points to a functional interaction between the UPR, TORC1, and CWI signaling pathways. Here, we use a hyperactive variant of *TOR1* to present a novel mechanism of ER stress regulation by TORC1 signaling. We show that attenuation of TORC1 signaling is required for adaptation to ER stress, and that hyperactive TORC1 signaling results in compromised cell wall architecture. Taken together, we propose that hyperactivation of TORC1 signaling alters cell wall composition, sensitizing cells to ER stress causing agents such as antifungal drugs.


A
FKS2
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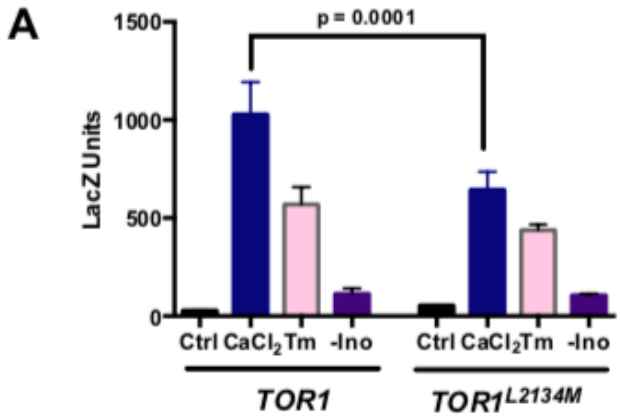
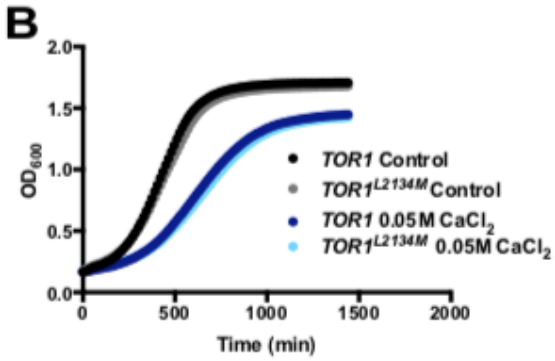
Ctrl
Tm
TOR1
TOR1L2134M
B
FKS1
p = 0.0016
p = 0.0256
C
PRM5
p = 0.0144
D
Control
0.1 µg/mL caspofungin
0.2 µg/mL caspofungin
TOR1
TOR1LM
E
IRE1
Ire1Δ

Figure 2.6: Cell wall perturbations in hyperactive *TOR1^{L2134M}* cells can be traced back to defects in glucan synthase activity

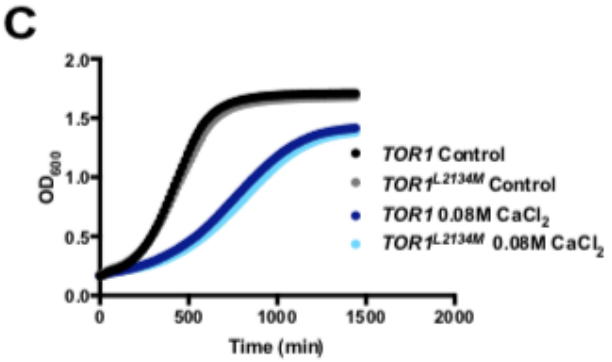
(A) Cells expressing *WT TOR1* or hyperactive *TOR1^{L2134M}* were treated with tunicamycin (Tm; 2.5 μg/mL) for 2 hrs. Tm induced a significant decrease in the expression of glucan synthase genes *FKS2* and **(B)** *FKS1* as measured by qRT-PCR (n=3; ± SD). **(C)** qRT-PCR was also used to assess the expression of the Rlm1 target, *PRM5* (n=3; ± SD). **(D)** Cell growth of WT *TOR1* and *TOR1^{L2134M}* cells was assessed by serial dilutions on YPD plates supplemented with various concentrations of the antifungal drug, caspofungin. Compared to WT TOR1, hyperactive *TOR1^{L2134M}* cells have impaired growth. **(E)** Growth of wild-type cells and *Ire1*Δ cells was assessed by serial dilutions on YPD plates supplemented with various concentrations of the antifungal drug, caspofungin. *Ire1*Δ cells have increased sensitivity to caspofungin.



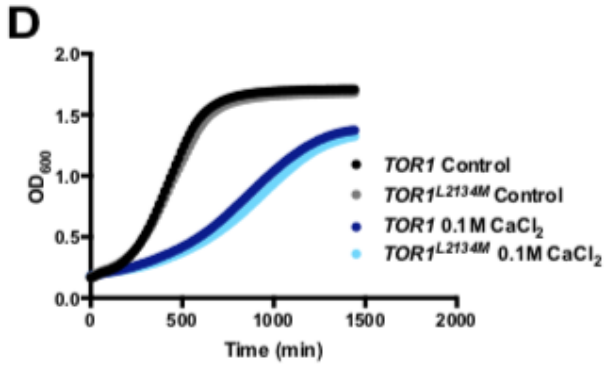


Control	AUC
TOR1	120.0 ± 0.5
TOR1^{L2134M}	116.7 ± 1.1

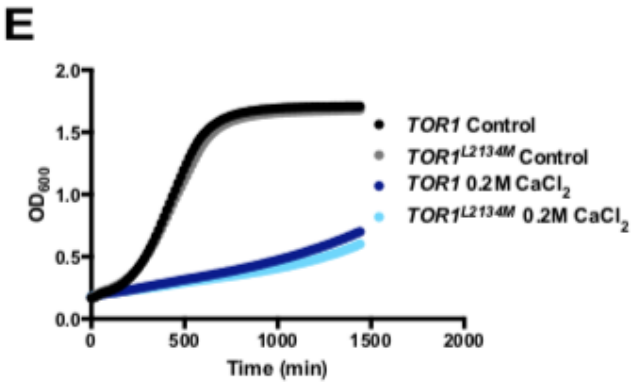
$CaCl_2$	AUC
TOR1	85.0 ± 0.5
TOR1^{L2134M}	82.5 ± 1.4



$CaCl_2$	AUC
TOR1	75.6 ± 0.5
TOR1^{L2134M}	71.2 ± 0.1



$CaCl_2$	AUC
TOR1	69.4 ± 0.4
TOR1^{L2134M}	69.4 ± 1.2



$CaCl_2$	AUC
TOR1	38.1 ± 0.5
TOR1^{L2134M}	34.0 ± 0.5

Figure 2.7: The Ca^{2+}/calcineurin pathway is not impaired in hyperactive *TOR1*^{L2134M} cells

(A) β-galactosidase activity (measured in LacZ units) was used to assess expression of calcineurin dependent response element (CDRE) following treatment with $CaCl_2$ (1 M), tunicamycin (Tm; 1.0 μg/mL), or inositol withdrawal (-ino; n =6). **(B)** Growth of cells expressing WT *TOR1* or hyperactive *TOR1*^{L2134M} was assessed by liquid growth assay following treatment with 0.05 M $CaCl_2$, **(C)** 0.08 M $CaCl_2$, **(D)** 0.1 M $CaCl_2$, or **(E)** 0. 2 M $CaCl_2$. The area under the curve (AUC) was quantified for each replicate (n=3). There was no significant difference ($p > 0.05$) between the two yeast strains.

2.3.5 Conclusion

The high prevalence of pathogenic fungal infections, coupled with the emergence of new fungal pathogens, has rapidly brought these diseases to the forefront of global health problem. Of particular concern are the millions of people worldwide that will contract life-threating invasive fungal infections (IFI) – diseases with a mortality rate which exceeds 50%, even with the availability of antifungal treatments [91,92]. As a whole, the aetiological agents responsible for more than 90% of IFI-related deaths fall largely within four genera of fungi: *Cryptococcus, Candida*, *Aspergillus*, and *Pneumocytis* [91,93]. While antifungal treatments have advanced over the last decade, patient outcomes have not substantially improved [94]. These shortcomings are largely attributed to the evolutionary similarity between fungi and humans, which limits the scope of drug development against fungal specific targets. As such, there is a pressing need to understand the unique cellular mechanisms that govern fungal viability. Given that *S. cerevisiae* is evolutionarily related to a number of pathogenic fungi, and in particular to the *Candida* species [95], most genes from *S. cerevisiae* are highly conserved in pathogenic fungal strains. Among the shared genomic features includes similar mechanisms for cell wall homeostasis [96–98] and activation of stress responses [99]. Here we show that hyperactivation of TORC1 signaling sensitizes yeast cells to both ER stress and cell wall stressors by compromising cell wall integrity. Therefore, targeting TORC1 signaling and ER stress pathways may be useful in developing novel targets for antifungal drugs.

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Chapter 3

3 General Overview

3.1 Discussion, Future Directions and Conclusions

The UPR is a major stress response pathway that is activated in response to an accumulation of misfolded proteins in the ER[1,2]. To mediate protein folding homeostasis, the UPR increases the protein folding capacity and decreases the protein folding load in the ER. While initially thought to function in isolation during ER stress, recent research indicates that the UPR is finetuned by signaling pathways from other cellular compartments. For instance, in *S.cereviasie*, both the Slt2 cell wall integrity pathway and the Hog1 hyperosmotic pathway have been implicated in the response to ER stress[3–5]. Furthermore, the transcriptional response to ER stress includes the induction of the general stress response (GSR) which is governed by PKA signaling and regulated by the Msn2/4 transcription factors[6,7]. Aside from its role in mediating the GSR, deactivation of PKA signaling during ER stress also decreases the protein folding load in the ER, namely by repressing ribosome biogenesis[8,9]. Given that downregulation of ribosome biosynthesis is a consequence of ER stress, we rationalized that exploring pathways involved in ribosomal homeostasis would allow us to elucidate key players that interface with the UPR to regulate ER homeostasis. Interestingly, in *S. cerevisiae*, the target of rapamycin complex 1 (TORC1) signaling pathway couples cell nutrient status and stress signaling to regulate ribosome biogenesis[10,11]. As such, elucidating the interplay between cell growth regulators, such as PKA and TORC1 signaling, and stress response pathways such as the UPR, is crucial in facilitating an understanding of the cellular response to proteotoxic stress.

Here we show that attenuating TORC1 signaling is required for adaptation to ER stress; however, what remains unclear are the upstream pathways responsible for propagating this signal to TORC1. Attenuation of TORC1 signaling during ER stress may be regulated by alterations in membrane composition[12,13]. In this regard, intracellular signaling pathways could be activated in response to changes in the physical properties of the plasma membrane such as fluidity or thickness[14]. More specifically, the impaired transport of membrane proteins and lipids from the secretory pathway, during ER stress, may trigger a signal to repress ribosome biogenesis in order to relieve secretory stress. Of particular interest are sphingolipids, molecules that not only function as second

messengers, but are also trafficked through the secretory pathway and delivered as integral components of the plasma membrane[14–16]. Recent research has proposed that sphingolipid/Pkh1/2-TORC1/Sch9 signaling may play a role in the transcriptional repression of ribosomal proteins, following tunicamycin-induced ER stress[17,18]. Nevertheless, further work is required to identify key sensors that impinge on TORC1 to attenuate downstream signaling during ER stress.

To determine how impacting proper TORC1 signaling affects the cell's response to ER stressors, we used a constitutive allele of the TOR1 kinase, *TOR1*^{L2134M}. We show that constitutive activation of TORC1 prevents its downregulation and confers increased sensitivity to ER stressors such as Tm and inositol withdrawal, independently of the UPR. We propose that TORC1 hyperactivation results in cell wall remodeling and impaired SWI4/6 signaling, and thereby sensitizes cells to ER stressors and cell wall antagonists, including the antifungal caspofungin (Fig. 3.1). This observation is particularly relevant to the field of pathogenic fungal infections, where there is a pressing need to identify novel targets for antifungal drugs. Interestingly, the components of the ER stress response pathways have been studied extensively in a number of fungal pathogens including the *Asperigullus* species[19–21], *Cryptoccous neoformans*[22,23], *Cryptococcus gattii*[24], *Candida albicans*[25], and *Candida galbrata*[26], with the basic aspects of the Ire1-mediated response being highly conserved amongst the species. However, while the ER stress response pathways play a redundant role in the virulence of most pathogenic species, there are some notable differences between species, particularly in *Candida glabrata*, where the Ire1-mediated ER stress response appears to be independent of *HAC1* splicing[26,27]. Therefore, it would be interesting to assess how these slight modifications in UPR signaling alter the response to ER stress signaling and whether hyperactivation of TORC1 signaling also sensitizes these pathogenic fungi to ER stress and antifungal drugs.

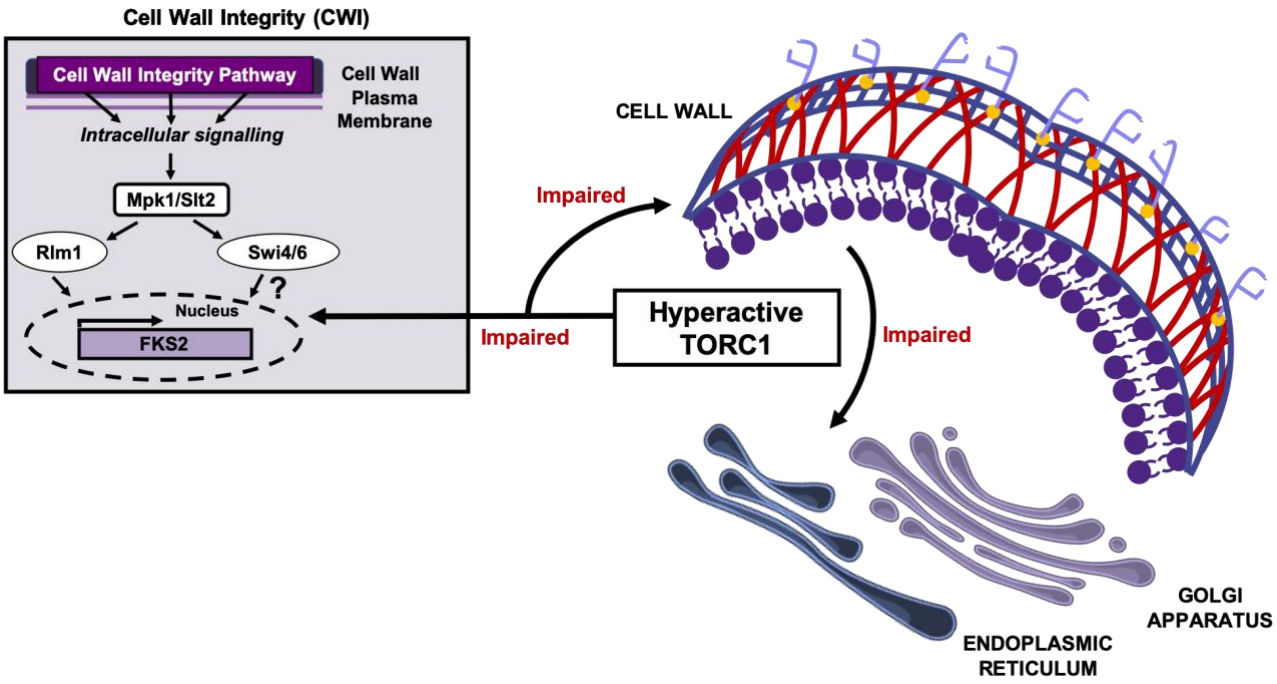
Cell Wall Integrity (CWI)
Cell Wall Integrity Pathway
Cell Wall
Plasma
Membrane
Intracellular signalling
Mpk1/Slt2
Rlm1
Swi4/6
?
Nucleus
FKS2
Impaired
Impaired
Hyperactive
TORC1
Impaired
CELL WALL
GOLGI
APPARATUS
ENDOPLASMIC
RETICULUM

Figure 3.1: Proposed model of the cellular response to hyperactive of TORC1

Hyperactive TORC1 signaling may compromise cell wall architecture by impairing Swi4/6 signaling. This disruption in cell wall integrity increases sensitivity to endoplasmic reticulum stress.

If hyperactivation of TORC1 signaling does sensitize pathogenic fungi to ER stress, another future direction of our study would be to design a therapeutic approach for TORC1 hyperactivation. Interestingly, previous studies in mammalian cells[28], *Drosophila*[29], *Schizosaccharomyces pombe*[30], and *Saccharomyces cerevisiae*[31] have implicated the Rag family of GTPases, Gtr1 and Gtr2, in the activation of TORC1 signaling. These studies indicate that Vam6, a GTP exchange factor (GEF), colocalizes with Gtr1 and TORC1 at the vacuole membrane and controls TORC1 by activating Gtr1[32–35]. Additionally, assessment of nucleotide restricted *GTR1* and *GTR2* alleles revealed that GTP-loaded Gtr1 and GDP-loaded Gtr2 stimulate TORC1 and GDP-loaded Gtr1 has a dominant negative phenotype[31]. Furthermore, GTP-loaded Gtr1 physically interacts with the Tco89 subunit of the TORC1 complex, suggesting that Gtr1 specifically controls TORC1 function[31]. Interestingly, overexpressing Vam6 rendered wild-type yeast cells resistant to low rapamycin concentrations and suppressed the semi-dominant growth defect resulting from GDP-loaded Gtr1[31]. Taken together, these results indicate that overexpressing the Vam6 GEF or inhibiting GTP hydrolysis may both be mechanisms to hyperactive TORC1 signaling in an antifungal context. Therefore, future experiments can assess the druggability of Vam6 or Gtr1 and examine them as potential activators of TORC1 signaling and novel targets for antifungal therapies.

Interestingly, the connection between TORC1 signaling and pathogenic fungal infections has been characterized through the use of the TORC1 inhibitor, rapamycin. In particular, rapamycin exerted growth inhibitory activity against *Mucor circinelloides* – an opportunistic fungal pathogen that infects patients with diabetes mellitus and solid organ transplants[36]. Similarly, other studies demonstrated that rapamycin has potent antifungal activity against *C. albicans* and *C. neoformans* through FKBP12-mediated inhibition of the Tor1 protein kinase[37]. As a whole, these observations suggest that TORC1 activation is required for fungal virulence, and that inhibition of TORC1 activity has potent antifungal effects. Additionally, our study in budding yeast demonstrated that TORC1 signaling needs to be properly regulated under conditions of cell stress and that inhibition of TORC1 signaling exacerbates toxicity to canonical ER stressors. Given that pathogenic fungi are constantly exposed to environmental stressors, exploring how

inhibition of TORC1 signaling affects cell stress response pathways may be an interesting avenue of study.

Conversely, recent findings in *C. albicans* support the model wherein the Tor1 protein kinase negatively regulates cellular adhesion[38]. Activation of Tor1 blocks cellular aggregation by promoting expression of adhesion transcriptional repressors, whereas inhibition of Tor1 activity, either during nutrient limiting conditions or rapamycin treatment, leads to expression of adhesion genes and formation of cellular aggregations, processes that are vital for *C. albicans* virulence traits such as niche colonization and biofilm secretion[38]. Taken together, these findings suggest that hyperactivation may also be a useful technique to inhibit virulence of pathogenic fungi. In essence, TORC1 activity may differentially mediate virulence traits in different fungal strains, such that inhibition of TOR may prove to be more efficacious against some fungal strains, whereas hyperactivation of TORC1 signaling may prove to have more portent antifungal activity in other strains. Nevertheless, the regulation of TORC1 signaling is an essential component of disease propagation, thus further work is required to examine how this nutrient-sensing pathway acts in parallel with ER stress response pathways to mediate cellular homeostasis.

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