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The role of Ire1 in azole sensitivity in Saccharomyces cerevisiae

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Abstract

In eukaryotes, the Unfolded Protein Response (UPR) maintains proteostasis in the endoplasmic reticulum (ER). In yeast, the UPR is activated by the ER-resident kinase/RNase sensor protein; Inositol requiring enzyme 1 (Ire1). During ER stress, Ire1 oligomerizes and splices the premature *HAC1* mRNA. The Hac1 transcription factor which binds to the unfolded protein response element (*UPRE*) and upregulate genes to mitigate the ER stress. Although poorly understood, the UPR is thought to play an essential role in antifungal resistance of pathogenic species. Therefore, with the highly characterized *Saccharomyces cerevisiae* model organism, I characterize the function of Ire1 upon azole treatment. For the first time in *S. cerevisiae*, I found that while the Ire1 is required for azole resistance, the UPR transcriptional response is dispensable as *HAC1* splicing and Ire1 oligomerization upon azole treatment do not occur. This suggests a requirement for Ire1 in azole resistance which is Hac1 and UPR independent. Moreover, I have found that the kinase and luminal domains of Ire1 are dispensable during azole resistance. I also show that the upregulation of the *UPRE*-regulated ERAD proteins; *KAR2* and *HRD1* are dispensable, suggesting the absence of proteotoxic stress upon azole treatment. Interestingly, I found that in the absence of Ire1, ergosterol synthesis genes which are regulated independently of the UPR are downregulated, which may explain the increased azole resistance in Δ*ire1* mutants. Overall, our data suggest that the *S. cerevisiae* Ire1 has a UPR independent function in the regulation of ergosterol synthesis genes which confers greater resistance to azoles.

Keywords

Saccharomyces cerevisiae, Azole drug resistance, UPR, Ire1, ERAD, Ergosterol

Lay Abstract

Antifungal drug resistance is increasingly becoming a more frequent cause of death by infection, especially in immunocompromised individuals. This is because, like the increase of antibiotic resistance in bacteria which require stronger and stronger antibiotics, the emergence of antifungal drug resistance in yeast have diminished the effectiveness of the limited selection of antifungals available today. Therefore, understanding the mechanisms used by yeast to develop resistance is vital for the creation of effective drugs. The current understanding of antifungal drug resistance is that stress adaptation pathways of these species are among the main contributing factors for the emergence of drug resistance in yeast. The day-to-day functions of the cell can often result in the formation of damaged proteins which must be corrected through stress adaptation pathways to resume normal cellular function. Unfortunately, upon treatment with antifungal drugs, these same pathways are activated to provide yeast cells drug resistance. Therefore, studying these pathways and how they help yeast become resistant antifungals will help develop new treatments and preserve the existing selection of drugs. In this study, with the use of baker's yeast, also known as *Saccharomyces cerevisiae*, I found for the first time that azole antifungal drug resistance requires the presence of a stress sensor protein, yet it does not activate its associated stress adaptation pathway. This suggests that this protein has an unknown function which confers yeast cells with drug resistance. Furthermore, I have found evidence to suggest that in the absence of this sensor protein, the synthesis of a yeast membrane component is reduced. Overall, our findings using *S. cerevisiae* suggest that azole resistance requires this sensor protein in a new way than previously described in literature.

Co-Authorship Statement

The *HAC1* and *HRD1* gene deletion strains and the DNA constructs pRS41K *IRE1* WT and pRS41K *ire1(*ΔLD) were created by Julie Genereaux, lab technician of Brandl and Lajoie Labs. With the exception of these, I performed all experiments presented in this thesis.

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List of Abbreviations

ATF-6	Activating transcription factor 6
DHE	Dehydroergosterol
DMSO	dimethyl sulfoxide
DTT	Dithiothreitol
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum Associated Degradation
HOG	High osmolarity glycerol
Ire1	Inositol requiring enzyme 1
Ire1-DX mutant	Ire1 diminished expression mutant
LBS	Lipid bilayer stress
MAPK	Mitogen activated protein kinase
MDR	Multi-drug resistance
PERK	pancreatic ER eIF2α kinase
RIDD	Regulated Ire1 dependent degradation
RNase	Ribonuclease
SC media	Synthetic Complete media
SRE	Sterol regulatory element
SRP	Signal recognition peptide
UPR	Unfolded protein response
UPRE	Unfolded protein response element
YPD media	Yeast extract Peptone Dextrose media

Chapter 1

1 Literature Review

1.1 Secretory Pathway Homeostasis

The endoplasmic reticulum (ER) is the largest membrane-bound organelle in eukaryotic cells which constitutes more than half of its membranous content and serves a myriad of purposes including lipid and steroid synthesis, carbohydrate metabolism, and protein synthesis. The ER membrane is organized into a netlike labyrinth of branching tubules and flattened sacs that extend throughout the cytosol to form a continuous sheet enclosing a single internal space known as the ER lumen (reviewed in (Alberts et al. 2002)). Moreover, as the ER membrane separates the cytosol and ER lumen, the ER membrane plays an important role in the selective transfer of molecules between these two compartments.

One crucial function of the ER is to serve as a site for protein synthesis for secreted and integral membrane proteins, as well as a subpopulation of cytosolic proteins. Of significance, approximately one-third of the yeast proteome enters the secretory pathway (Barlowe and Miller 2013). Firstly, the mRNA and ribosomal subunits form a complex which initiates translation of the nascent polypeptide in the cytosol (Schwarz and Blower 2016) (Figure 1.1 A). The mRNAs which encode secreted or integral membrane proteins, also contain an ER signal sequence which co-translationally is bound by the signal recognition peptide (SRP) (P. Walter and Blobel 1981; P. Walter, Ibrahimi, and Blobel 1981). Then, the mRNA:ribosome:nascent polypeptide:SRP complex is localized to the cytosolic face of the ER membrane by docking on the SRP receptor residing in the ER membrane (G. Reid, Blobel, and Walter 1982; Meyer, Krause, and Dobberstein 1982). The SRP receptor then guides the ribosome complex to dock with the ER-translocon (Jiang et al. 2008, 200), which results in the partial translocation and embedding of the peptide across the ER membrane (for integral/transmembrane proteins) (Blobel 1980), or completely translocated into the ER lumen (for secreted proteins and ER lumen chaperone proteins) (Evans, Gilmore, and

Blobel 1986). The translocon is a channel protein that contains several Sec proteins and spans the ER lipid bilayer (Deshaies et al. 1991). Ribosomes with mRNA for ER bound proteins engage in synthesis of peptides that are co-translationally translocated into the ER and is therefore directly bound to the surface of the ER, creating regions known as rough ER (D. W. Reid and Nicchitta 2012; Jagannathan et al. 2014). Alternatively, ribosomes bound to mRNA that do not contain an ER signal sequence, synthesize peptides unattached to the ER membrane and release the nascent proteins into the cytosol (Palade 1955) (Figure 1.1 B.). ER bound and free ribosomes are structurally and functionally identical, differing only in the proteins they are making at any given time (Alberts et al. 2002). Upon completion of protein synthesis, the ribosomal subunits are dissociated and recycled back into the cytosol for further mRNA translation.

The lumen of the ER is a specialized environment for the proper folding of proteins which not only has an oxidizing environment to create favorable conditions for disulfide bond formation, but also contains multiple chaperones such as yeast Kar2 (GRP78/BiP in mammals) to facilitate proper folding and help minimize misfolded/unfolded protein aggregation (Araki and Nagata 2011; Braakman, Helenius, and Helenius 1992). Despite this, protein misfolding can still occur due to normal physiological and pathological conditions such as translational errors, thermal or oxidative stress, nutrient deprivation, and high protein demand due to increased protein synthesis (Zhang and Wang 2012; Chambers and Marciniak 2014; Oslowski and Urano 2011; Cao and Kaufman 2012). These ER stress conditions dysregulate ER homeostasis and increase the likelihood of misfolded proteins. If ER stress persists, aberrant proteins can accumulate, which is detrimental for the cell. Therefore, the efficiency of protein folding is constantly monitored and adjusted through the integration of multiple proteotoxic stress adaptation pathways such as the ER quality control (ERQC) mechanism which facilitates folding and modification of secretory and

membrane proteins (Caldwell, Hill, and Cooper 2001). Moreover, terminally misfolded polypeptides are eliminated through ER-associated degradation (ERAD) or autophagic degradation, thereby ensuring that only properly folded proteins are trafficked through the Golgi apparatus (Araki and Nagata 2011). To mitigate the accumulation of misfolded proteins that exceeds the capacity of the ER quality control machinery, a network of intracellular signaling and transcriptional events are initiated which are collectively known as the unfolded protein response (UPR) (Peter Walter and Ron 2011a; H. Wu, Ng, and Thibault 2014a).

In addition to physiological and pathological conditions, pharmacological compounds such as tunicamycin and dithiothreitol (DTT) can also induce proteotoxic stress and have therefore been used extensively to characterize the cellular response to ER stress. Tunicamycin acts by inhibiting the UDP-GlcNAc-phosphate transferase which essentially blocks the N-linked glycosylation step of protein synthesis (Zhu, Zeng, and Lehrman 1992). DTT is a potent reducing agent which disrupts the formation of disulfide bonds between cysteine residues (Braakman, Helenius, and Helenius 1992; Kaji and Lodish 1993; Schröder and Kaufman 2005). Therefore, treatment with either tunicamycin or DTT leads to the accumulation of misfolded proteins within the ER lumen which necessitates ER stress pathways such as ERAD and UPR. Indeed, it is well known that *S. cerevisiae* with functional ER stress adaptation pathways are able to mediate and clear the stress caused by low concentrations of these proteotoxic stressors (Ho et al. 2020a).

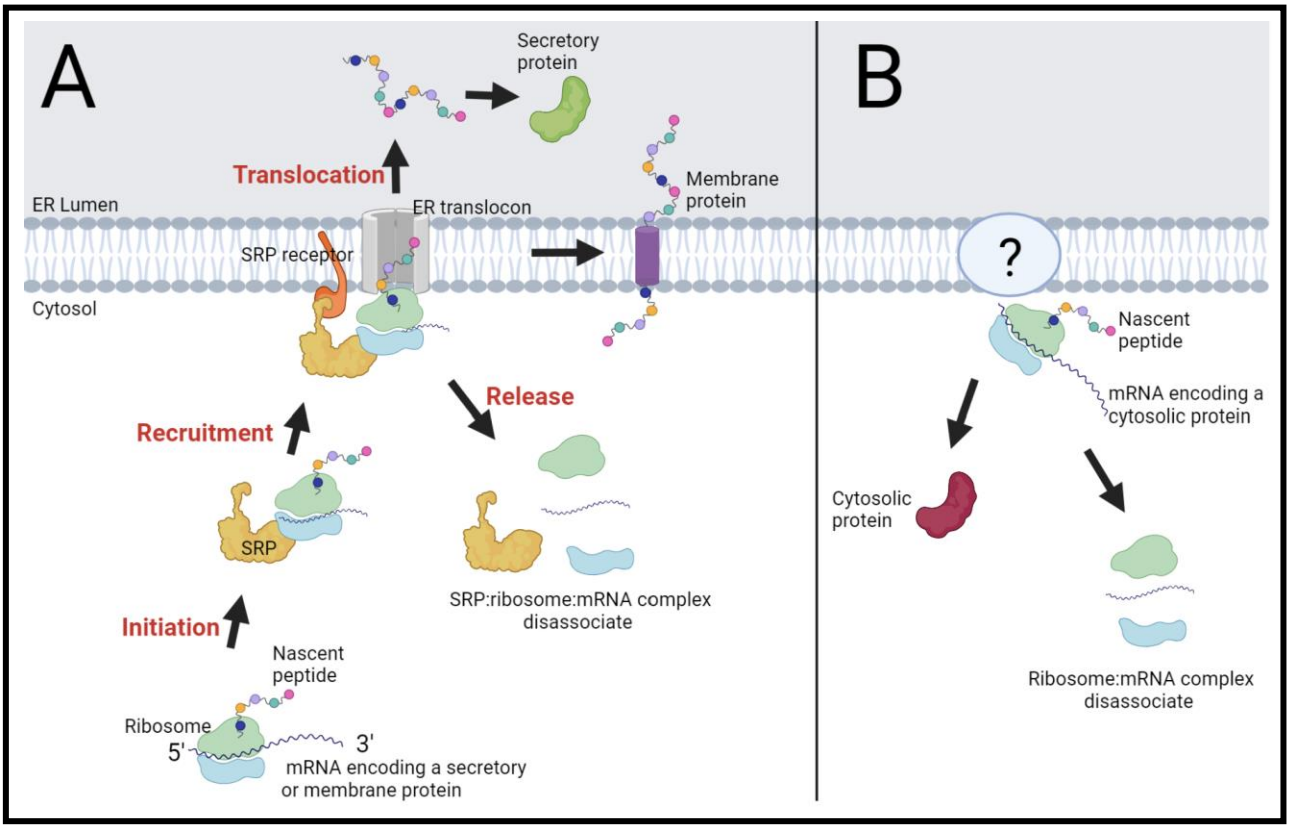

A
Secretory protein
Translocation
ER Lumen
ER translocon
Membrane protein
SRP receptor
Cytosol
Release
Recruitment
SRP
SRP:ribosome:mRNA complex disassociate
Initiation
Nascent peptide
Ribosome
5'
3'
mRNA encoding a secretory or membrane protein
B
?
Nascent peptide
mRNA encoding a cytosolic protein
Cytosolic protein
Ribosome:mRNA complex disassociate

Figure 1.1: Depiction of secretory and cytosolic protein synthesis.

A. Schematic of the generally accepted model for secretory protein translation. B. Schematic of the model for cytosolic protein translation. Image created with BioRender.

1.2 The Unfolded Protein Response

ER integrity and proper folding of proteins are crucial for cellular homeostasis, and the accumulation of misfolded proteins is detrimental to the cell. Cellular stresses in the form of toxic chemicals, radiation, changes in temperature, and genetic mutations can lead to aberrant accumulation of misfolded proteins. Thus, to maintain cellular protein homeostasis or proteostasis of secretory proteins in the ER, eukaryotic cells have evolved a highly conserved stress detection and recovery system known as the Unfolded Protein Response (UPR) (Peter Walter and Ron 2011a; H. Wu, Ng, and Thibault 2014a). The UPR restores ER homeostasis through 3 mechanisms, 1) attenuation of protein synthesis to reduce protein load within the ER lumen, 2) transcriptional activation of UPR genes to increase folding capacity, and 3) stimulation of the ER membrane to expand ER volume (Cox, Chapman, and Walter 1997; Cox and Walter 1996; Ron and Walter 2007). If the ER stress persists and cannot be mitigated, constitutive UPR can lead to cell death through apoptosis (Szegezdi et al. 2006; Rubio et al. 2011; Chawla et al. 2011).

The UPR signaling pathway in metazoans is quite complex and can be initiated by 3 distinct UPR sensor proteins: inositol requiring enzyme 1 (*IRE1*), pancreatic ER eIF2α kinase (*PERK*), and activating transcription factor 6 (*ATF*-6). Although the UPR can be activated by all three branches in eukaryotes like mammals, in lower eukaryotes such as yeast, only the Ire1 branch is present (H. Wu, Ng, and Thibault 2014b). Indeed, the UPR was initially discovered in the yeast *Saccharomyces cerevisiae* and as such, the Ire1 branch is highly characterized in this species and was found to be the most evolutionarily conserved branch of the UPR. Ire1 consists of a luminal domain that detects proteotoxic stress within the ER lumen by directly binding to misfolded proteins (Peter Walter and Ron 2011b), a single-pass transmembrane domain that detects Lipid Bilayer Stress (LBS) (Ho et al. 2020a) and interacts with other Ire1 monomers (Korennykh et al.

2009), and finally a cytoplasmic domain which is further divided into a kinase domain and a ribonuclease (RNase) domain (Mori et al. 1993, 19; Sidrauski and Walter 1997) (Figure 1.2). The canonical trigger for Ire1 activation in yeast is an accumulation of misfolded proteins within the ER lumen, and there are two proposed models for the activation of UPR via proteotoxic stress (Read and Schröder 2021) (Figure 1.3). One model suggests that the Ire1 luminal domain (which contains two interfaces) directly binds to misfolded proteins (Credle et al. 2005). The first interface creates a deep grove that is thought to bind misfolded proteins with high specificity. The second interface allows Ire1 oligomerization and subsequent activation (Credle et al. 2005; B. M. Gardner and Walter 2011). The second proposed model involves the ER chaperone protein Kar2 which is bound to inactive Ire1 (Kimata et al. 2003; Pincus et al. 2010), and preferentially binds misfolded proteins when they accumulate (Read and Schröder 2021; Armstrong et al. 2017; Lee et al. 2008; Shamu and Walter 1996). The release of Kar2 from Ire1 is thought to allow Ire1 to oligomerize (Kimata et al. 2003; Pincus et al. 2010). In both models, subsequent activation occurs through Ire1 clustering and autophosphorylation of the kinase domain, which enhances the endonuclease activity of the RNase domain (Shamu and Walter 1996; Lee et al. 2008; Armstrong et al. 2017). However, it should be noted that the kinase domain is dispensable for Ire1 activation yet is required for Ire1 attenuation (Rubio et al. 2011). The RNase domain functions by removing an intron of 252-nucleotides in the constitutively expressed *HAC1* mRNA to produce the mature spliced form (Sidrauski, Cox, and Walter 1996). The splicing of *HAC1* is unique, as most other pre-mRNAs are modified within the nucleus by the spliceosome. In contrast, the extranuclear splicing of *HAC1* mRNA is somewhat like pre-tRNA splicing (Sidrauski, Cox, and Walter 1996). Once the mature *HAC1* is translated and localized to the nucleus, this transcription factor binds to *UPRE*s upstream of various genes (Nikawa et al. 1996; Niwa et al. 1999), through its DNA binding region and

leucine zipper motif (Mori et al. 1996). Interestingly, it has been proposed that Hac1 binds to long and short versions of the *UPRE* promoter, thereby allowing transcriptional regulation of a wide variety of genes encoding proteins that contribute to the UPR (Fordyce et al. 2012). Specifically, Hac1 is known to transcriptionally regulate the expression of approximately 400 genes that are responsible for mediating ER protein folding, quality control, ERAD, membrane expansion, lipid homeostasis, and cell wall integrity, among others (Xia 2019; Lu et al. 2014; Ghosh et al. 2014, 1; Rutkowski and Kaufman 2007; Rutkowski et al. 2006; H. Wu, Ng, and Thibault 2014a; Travers et al. 2000).

In addition to the accumulation of aberrant proteins within the ER lumen, recent studies show that changes in the ER membrane composition can also lead to Ire1 oligomerization resulting in UPR activation, which specifically results in lipid biosynthetic gene regulation (Lajoie et al. 2012, 2; Promlek et al. 2011; Volmer and Ron 2015; Volmer, van der Ploeg, and Ron 2013; Ho et al. 2020b; Koh et al. 2018). Moreover, it has been shown that proteotoxic stress and changes to the lipid membrane are detected by distinct Ire1 mechanisms thereby activating distinct transcriptional responses (Ho et al. 2020b).

In species such as *Candida glabrata*, an additional function of Ire1 has been characterized whereby Ire1 contributes to targeted mRNA degradation, which is known as regulated Ire1 dependent degradation (RIDD) (Kimmig et al. 2012; Hernández-Elvira et al. 2018). Currently, in *S, cerevisiae*, the only characterized function of Ire1 upon ER stress, is the indirect activation of the UPR transcriptional response through excision of the *HAC1* intron (reviewed in (Read and Schröder 2021)). Indeed, there has been no evidence of RIDD documented in *S. cerevisiae*. Moreover, in *S. cerevisiae*, the only known substrate of Ire1 is *HAC1* (Kimmig et al. 2012; Hernández-Elvira et al. 2018). Interestingly, unlike in *S. cerevisiae*, the Ire1 of

Schizosaccharomyces pombe is known to exclusively rely on RIDD under conditions of ER stress (Kimmig et al. 2012). RIDD works to reduce the load of nascent protein within the ER by degrading mRNAs that are localized to the ER so that the ER lumen may have an opportunity to mitigate the already present misfolded proteins (Kimmig et al. 2012; Hernández-Elvira et al. 2018). The cleavage site of the RIDD targeted mRNAs are similar to the cleavage sequence of the *HAC1*, then the mRNA fragments are degraded by exoribonucleases (Maurel et al. 2014).

Although deletion of the genes *IRE1* and *HAC1*, which abolish the UPR are viable in yeast, they are required for survival upon ER stress (Cox, Chapman, and Walter 1997; Chen et al. 2005). Interestingly, as antifungal drugs often target the ER, the UPR has been implicated in the emergence of antifungal drug resistance in pathogenic yeast species (reviewed in (Krishnan and Askew 2014)) and will be discussed in section 1.5. Moreover, in this study I determined the importance of the Hac1-dependent UPR transcriptional response and function of Ire1 during azole drug resistance.

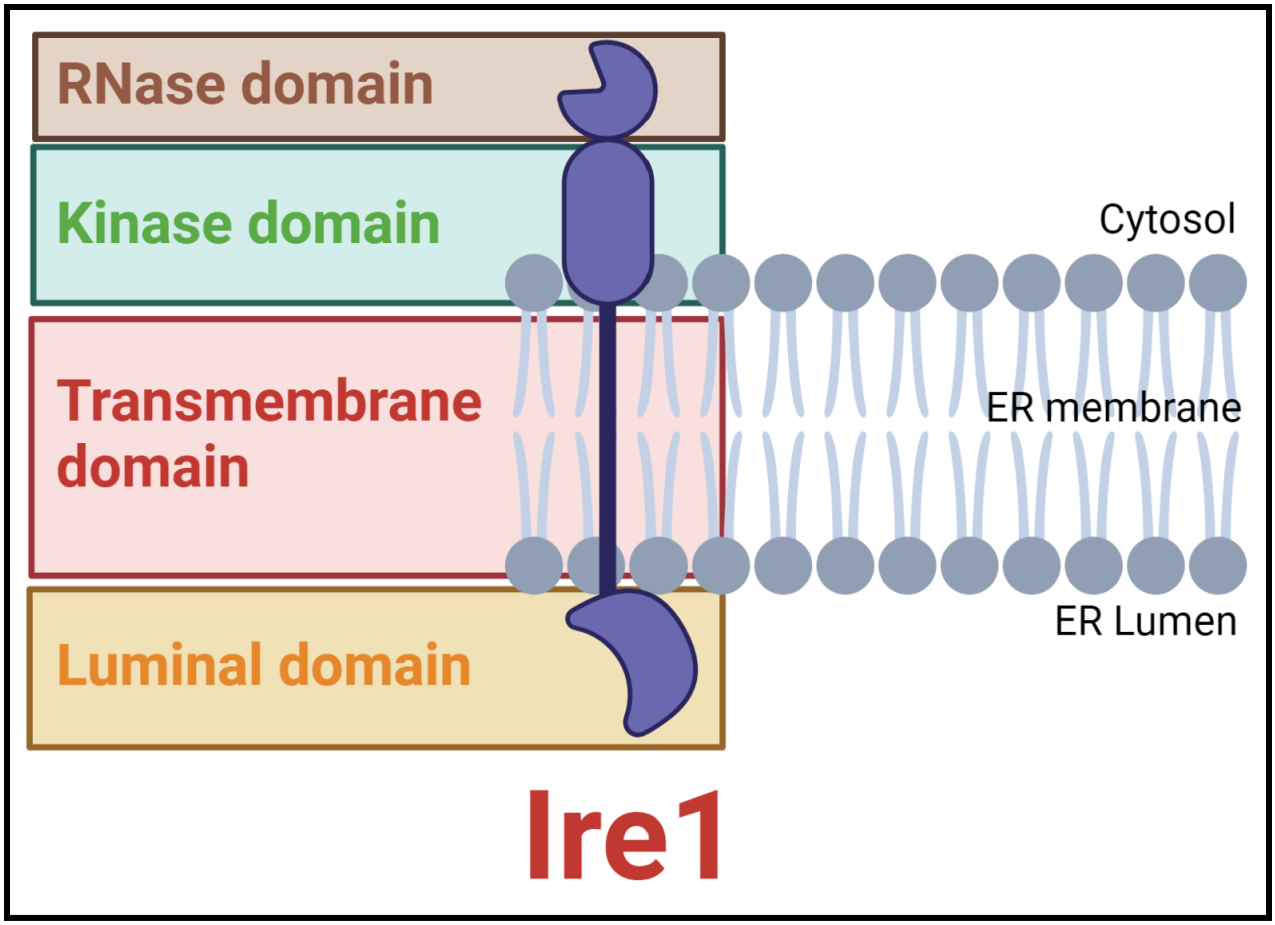

RNase domain
Kinase domain
Transmembrane domain
Luminal domain
Cytosol
ER membrane
ER Lumen
Ire1

Figure 1.2: Schematic of the Ire1 protein.

The Ire1 protein is an integral protein of the ER membrane. The RNase and kinase domains are located in the cytosolic side of the ER membrane. The transmembrane domain is embedded within the ER membrane, and the luminal domain is located within the ER lumen. Figure created with BioRender.

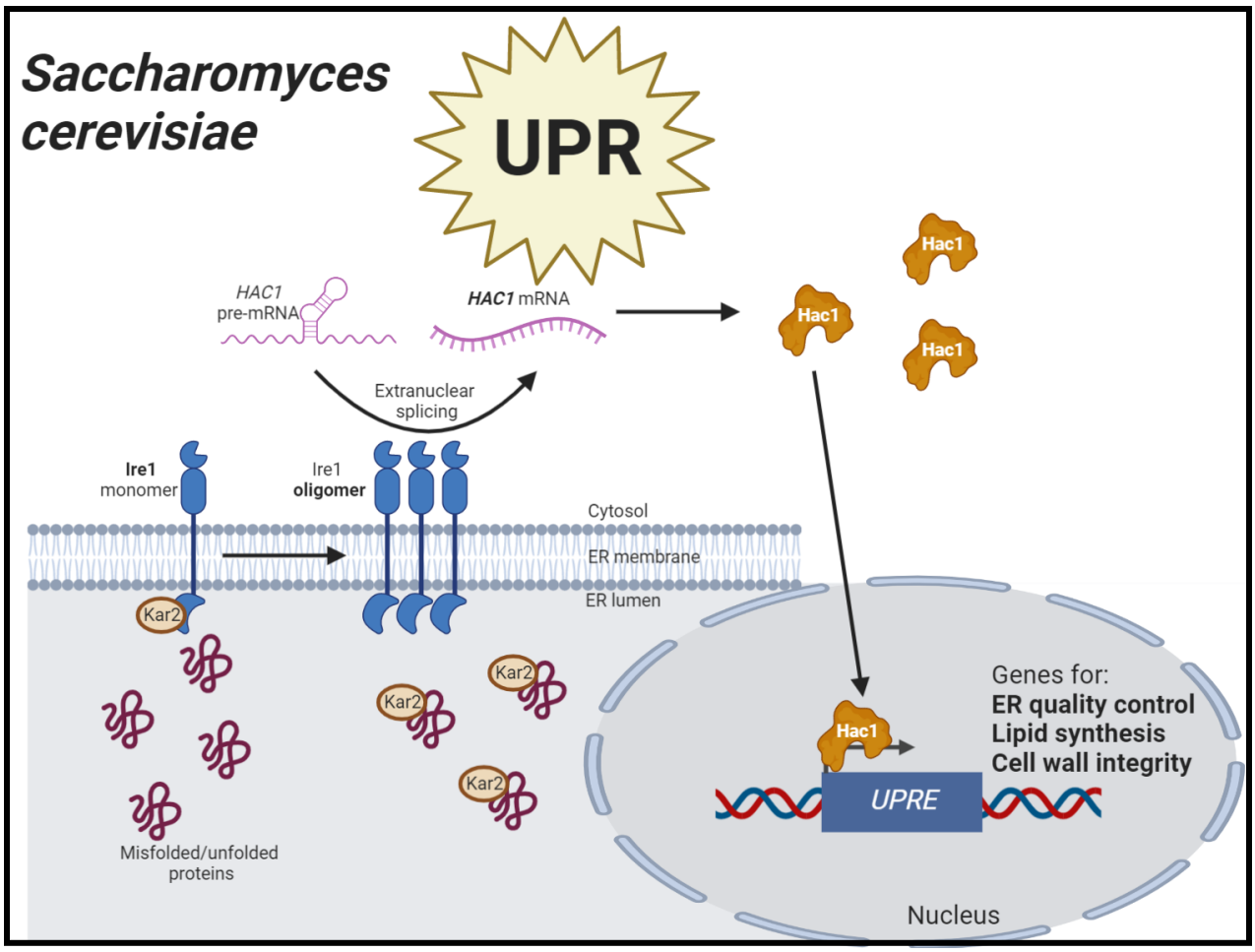

Saccharomyces cerevisiae
UPR
HAC1 pre-mRNA
HAC1 mRNA
Hac1
Hac1
Hac1
Extranuclear splicing
Ire1 monomer
Ire1 oligomer
Cytosol
ER membrane
ER lumen
Kar2
Kar2
Kar2
Kar2
Misfolded/unfolded proteins
Hac1
UPRE
Genes for:
ER quality control
Lipid synthesis
Cell wall integrity
Nucleus

Figure 1.3: Unfolded protein response in Saccharomyces cerevisiae.

Unfolded/ misfolded proteins detected by the luminal domain of Ire1 results in its oligomerization. This leads to the extranuclear splicing of premature *HAC1* mRNA to the mature form. Translated Hac1 proteins localize to the nucleus to upregulate genes that mediate correct protein folding. Figure adapted from (Lajoie, Fazio, and Snapp 2014), and created with BioRender.

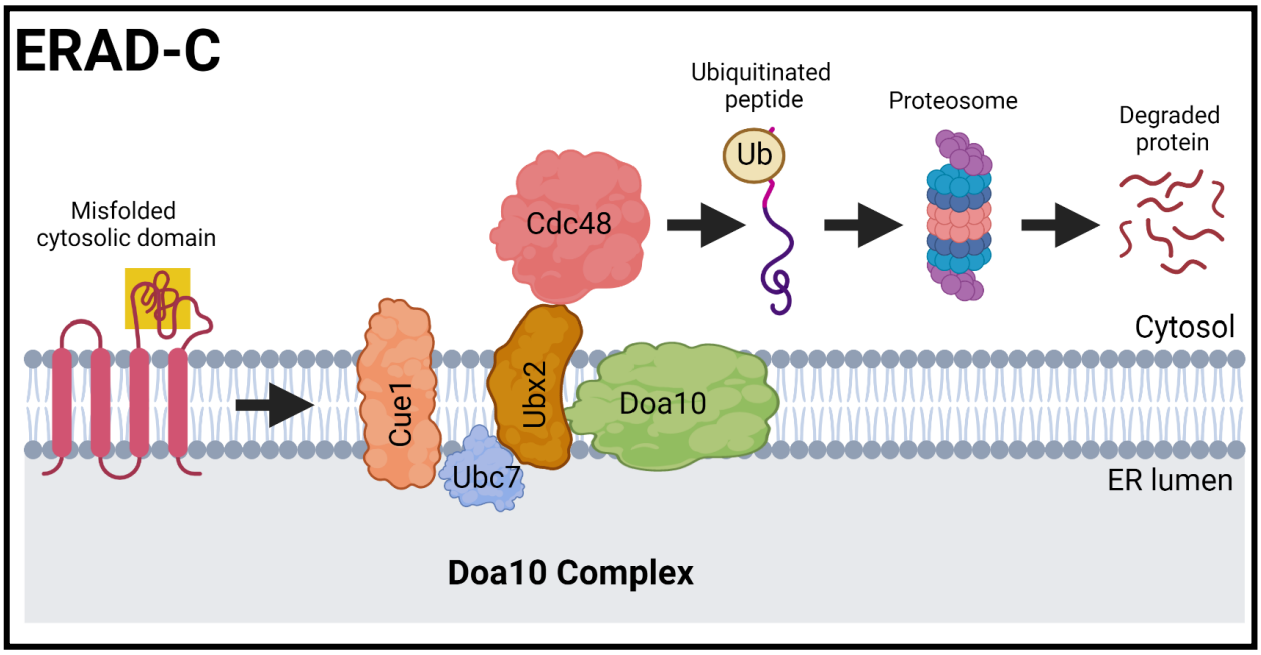
1.3 ER-Associated Degradation (ERAD)

While the UPR is activated in response to the accumulation of misfolded proteins, the ERAD pathway is directly responsible for the detection and clearance of misfolded proteins within the ER (X. Wu and Rapoport 2018; Satpute-Krishnan et al. 2014). Misfolded proteins are removed from the ER in an evolutionarily conserved process which detects and retro-translocates substrates into the cytosol, leading to the polyubiquitination and cytosolic proteasomal degradation (Christianson and Ye 2014). Although most ERAD substrates follow this general scheme, distinct pathways are used depending on which domain of a protein is misfolded (Huyer et al. 2004; Vashist and Ng 2004). Proteins with misfolded cytosolic domains (ERAD-C substrates) require the ubiquitin ligase Doa10 (Swanson, Locher, and Hochstrasser 2001) (Figure 1.4). Proteins with misfolded luminal domains (ERAD-L substrates) are detected by the luminal chaperones Yos9 and Kar2 (Kim, Spear, and Ng 2005, 9; Mehnert et al. 2015) and may cycle through the cis-Golgi compartment before being retro-translocated to the cytoplasm (Vashist and Ng 2004; Caldwell, Hill, and Cooper 2001) (Figure 1.4). The ERAD-L pathway also requires the ubiquitin ligase Hrd1 for substrate polyubiquitylation (Bays et al. 2001; Bordallo et al. 1998) which associates with other membrane proteins Hrd3, Usa1, Der1 and the luminal chaperone Yos9 (Bays et al. 2001; Bordallo et al. 1998; R. G. Gardner et al. 2000; Carvalho, Goder, and Rapoport 2006). Proteins with misfolded transmembrane domains (ERAD-M substrates) also require Hrd1 (which detects lesions of the membrane domain), and Hrd3, but not Der1 (Carvalho, Goder, and Rapoport 2006), and in some cases Usa1 (Horn et al. 2009, 1) (Figure 1.4). The Hrd1 protein is especially important as it is thought to be the retro-translocation channel (Ye et al. 2004; Lilley and Ploegh 2004) whose overexpression in yeast makes other components of the complex dispensable for the degradation of ERAD-L-and -M substrates (R. G. Gardner et al. 2000; Carvalho, Stanley, and Rapoport 2010;

Plemper et al. 1999). Once the ERAD-L substrate inserts into the Hrd1 membrane channel from the ER lumen (Denic, Quan, and Weissman 2006; Gauss et al. 2006), it is polyubiquitinated on the cytoplasmic side of the ER membrane by the Hrd1 (Bays et al. 2001; Bordallo et al. 1998; Stein et al. 2014). ERAD-M substrates most likely enter the Hrd1 channel from the side (X. Wu and Rapoport 2018; Knop et al. 1996; Mehnert, Sommer, and Jarosch 2014). Once the misfolded/unfolded proteins have begun to retro-translocate through any of ERAD – C, L, or M pathways, the Cdc48 ATPase complex is recruited to the membrane (Neuber et al. 2005; Schuberth and Buchberger 2005) and uses the energy of ATP hydrolysis to progressively pull the substrate out of the membrane (Stein et al. 2014; Bodnar and Rapoport 2017). Finally, the substrate is passed on to the proteosome for degradation (X. Wu and Rapoport 2018).

Since both the UPR and ERAD function to monitor and maintain a proper protein folding environment, crosstalk between these quality control systems occur (Hwang and Qi 2018). For example, Hac1 is responsible for the transcription of some ERAD machinery genes such as Kar2 and Hrd1 (Ho et al. 2020b) (also reviewed in (Hwang and Qi 2018; Read and Schröder 2021)) . Furthermore, studies in mammalian cell models have found that under standard conditions the IRE1α (the mammalian orthologue of Ire1) is a substrate for the ERAD (Sun et al. 2015; Hwang and Qi 2018). However, under ER stress, the mammalian Kar2 homologue known as BiP and the HRD1 complex are released from IRE1α, thereby attenuating its degradation, and promoting stability. Moreover, ERAD deficiency was found to cause IRE1α stabilization, accumulation, and mild activation both in vitro and in vivo during standard conditions due to normally expressed misfolded proteins not being degraded. Interestingly, it has also been found that in *S. cerevisiae,* during LBS which activates the UPR, premature removal of key ER-resident transmembrane proteins occurs through the Doa10 complex (Shyu et al. 2019). In this study, I attempt to determine

if azole drug resistance requires the Hrd1-dependent ERAD by utilizing *KAR2* and *HRD1* deletion mutants.


ERAD-C
Ubiquitinated peptide
Proteosome
Degraded protein
Misfolded cytosolic domain
Cdc48
Ub
Cytosol
Cue1
Ubx2
Doa10
Ubc7
ER lumen
Doa10 Complex

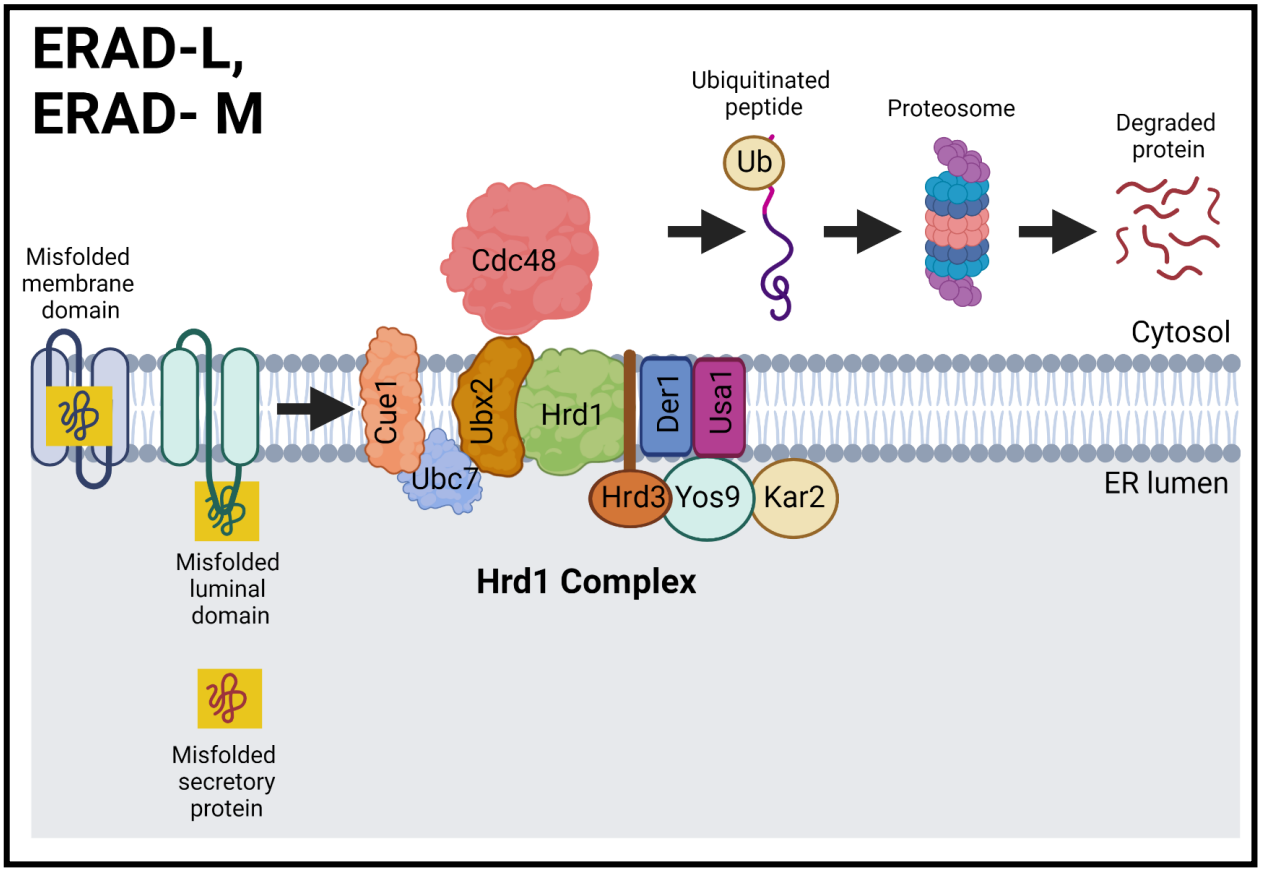

ERAD-L,
ERAD- M
Ubiquitinated peptide
Proteosome
Degraded protein
Misfolded membrane domain
Cdc48
Ub
Cytosol
Cue1
Ubx2
Hrd1
Der1
Usa1
Ubc7
Hrd3
Yos9
Kar2
ER lumen
Misfolded luminal domain
Hrd1 Complex
Misfolded secretory protein

Figure 1.4 Schematic representations of ERAD-C, L and M.

Individual subunits of the Doa10 and Hrd1 complexes are depicted with some of their known partners. (*Top box*) the Doa10 complex monitors the folding state of cytosolic domains of membrane proteins (ERAD-C). (*Bottom box*) The Hrd1 complex recognizes luminal domain lesions of soluble and transmembrane proteins (ERAD-L) as well as lesions within the membrane domains (ERAD-M). Figure adapted from (Thibault and Ng 2012), and created with BioRender.

1.4 Ergosterol biosynthesis and regulation

Sterols are essential components of the cellular membrane of all eukaryotic cells as they help maintain membrane structural integrity, fluidity, and permeability. In yeast, the main fungal sterol is ergosterol, and its synthesis is tightly controlled by the bioavailability of certain metabolites (oxygen, iron, and sterols) and environmental conditions (Shakoury-Elizeh et al. 2010; Kwast et al. 1999; Jordá and Puig 2020). In fact, oxygen acts as the electron acceptor in the enzymatic steps catalyzed by Erg1, Erg11, Erg25, Erg3, and Erg5 (Jordá and Puig 2020); and iron is required for the heme groups of the cytochrome enzymes Erg11, Erg5, Erg 25, and Erg3 (Liu et al. 2019; Ward et al. 2018) (Figure 1.5).

Ergosterol synthesis occurs through a highly conserved pathway that can be divided into 3 modules (reviewed in (Hu et al. 2017)). The first module occurs in the vacuole and mitochondria, and comprises the enzymatic reactions required for the conversion of acetyl-coenzyme A (acetyl-CoA) into mevalonate. The second module results in the formation of farnesyl pyrophosphate (farnesyl-PP) within the vacuole. Finally, the third module or late pathway in which ergosterol is biosynthesized, mainly occurs through a series of consecutive reactions within the ER. Therefore, many of the ergosterol biosynthesis enzymes in the late pathway are localized to the ER. Indeed, the transmembrane enzymes Erg11, Erg25, Erg27, and Erg28 associate into a functional complex denoted as the ergosome within the ER membrane (Mo and Bard 2005). Erg24, Erg2, Erg3, Erg4, and Erg5 are also primarily located in the ER (Zweytick et al. 2000; Kristan and Rižner 2012). Other enzymes such as Erg1, Erg7, Erg27, and Erg6 localize to both the ER and lipid particles, which are not only storage compartments for triacylglycerols, but also function to store and synthesize sterols (Müllner et al. 2004; Kristan and Rižner 2012). Interestingly, despite the ER being the primary site of ergosterol synthesis, about 70 % of the ergosterol is transported to the

plasma membrane, mostly contained within the cytoplasmic leaflet. The ER on the other hand contains only 10-30 % of the total ergosterol content of the cell (van der Rest et al. 1995; Schneiter et al. 1999; Zinser and Daum 1995; Solanko et al. 2018).

The biosynthesis of ergosterol is very energy consuming; requiring 24 molecules of ATP and 16 molecules of NADPH to obtain a single molecule of ergosterol (reviewed in (Hu et al. 2017)). Despite this, under aerobic conditions, yeast cells do not incorporate exogenous sterols, and instead synthesize their own to ensure that only the highest quality sterols are incorporated into the plasma membrane (Lorenz and Parks 1987; Hughes, Todd, and Espenshade 2005; Zavrel, Hoot, and White 2013). Under anaerobic conditions, the decreased ability to synthesize sterols is mitigated by the import of exogenous sterols (Hu et al. 2017). However, upon return to aerobic conditions, the exogenous sterols incorporated into the plasma membrane are replaced by new endogenously synthesized ergosterol (Georgiev et al. 2011), thereby indicating the importance of the tight regulation and synthesis of ergosterol.

Ergosterol biosynthesis is transcriptionally controlled by certain stress adaptation pathways such as the high osmolarity glycerol (HOG) pathway which is responsible for adaptation of hyperosmotic stress (Montañés, Pascual-Ahuir, and Proft 2011). Interestingly, through the use of microarray analysis, the UPR transcriptional response upon ER stress (both proteotoxic and LBS) in *S. cerevisiae* was not found to regulate any ergosterol synthesis genes (Ho et al. 2020b). The HOG pathway is a mitogen activated protein kinase (MAPK) pathway that regulates hyperosmotic stress adaptation. Upon osmotic stress, the MAPK Hog1 is activated and localizes to the nucleus (reviewed in (Miermont et al. 2011)). Then the phosphorylated Hog1 proceeds to regulate various genes for stress adaptation, including the regulation of ergosterol synthesis genes through the transcription regulators *UPC2*, *ECM22*, *ROX1*, and *MOT3* that bind the sterol regulatory element

(*SRE*) (Montañés, Pascual-Ahuir, and Proft 2011). Indeed, over production of ergosterol in yeast works to increase tolerance to certain stressors such as antifungal drugs, low temperatures, freezing, alcohol, low sugar, hypo-osmotic conditions, and oxidative tress (Jordá and Puig 2020; Hu et al. 2017; Montañés, Pascual-Ahuir, and Proft 2011). Conversely, without the addition of exogenous ergosterol, deletion of most *ERG* genes within the late pathway is detrimental to the cell. In fact, it was found that *erg* mutants are defective in certain cellular processes such as endocytosis, cell polarization, cell fusion, cell wall assembly, and show alterations in resistance to certain stresses (reviewed in (Jordá and Puig 2020; Joshua and Höfken 2017)). Therefore, due to the importance of ergosterol in yeast stress regulation, the main target of many of the antifungal drugs available today are the enzymes of the late ergosterol biosynthetic pathway (indicated in Figure 1.5). Importantly, the most common antifungal drug class in use today are the Azoles, which function by inhibiting the Erg11 transmembrane enzyme localized to the ER membrane. Furthermore, as the UPR is important in antifungal drug resistance (reviewed in (Krishnan and Askew 2014)), I explore the link between ergosterol regulation and the UPR sensor protein Ire1.

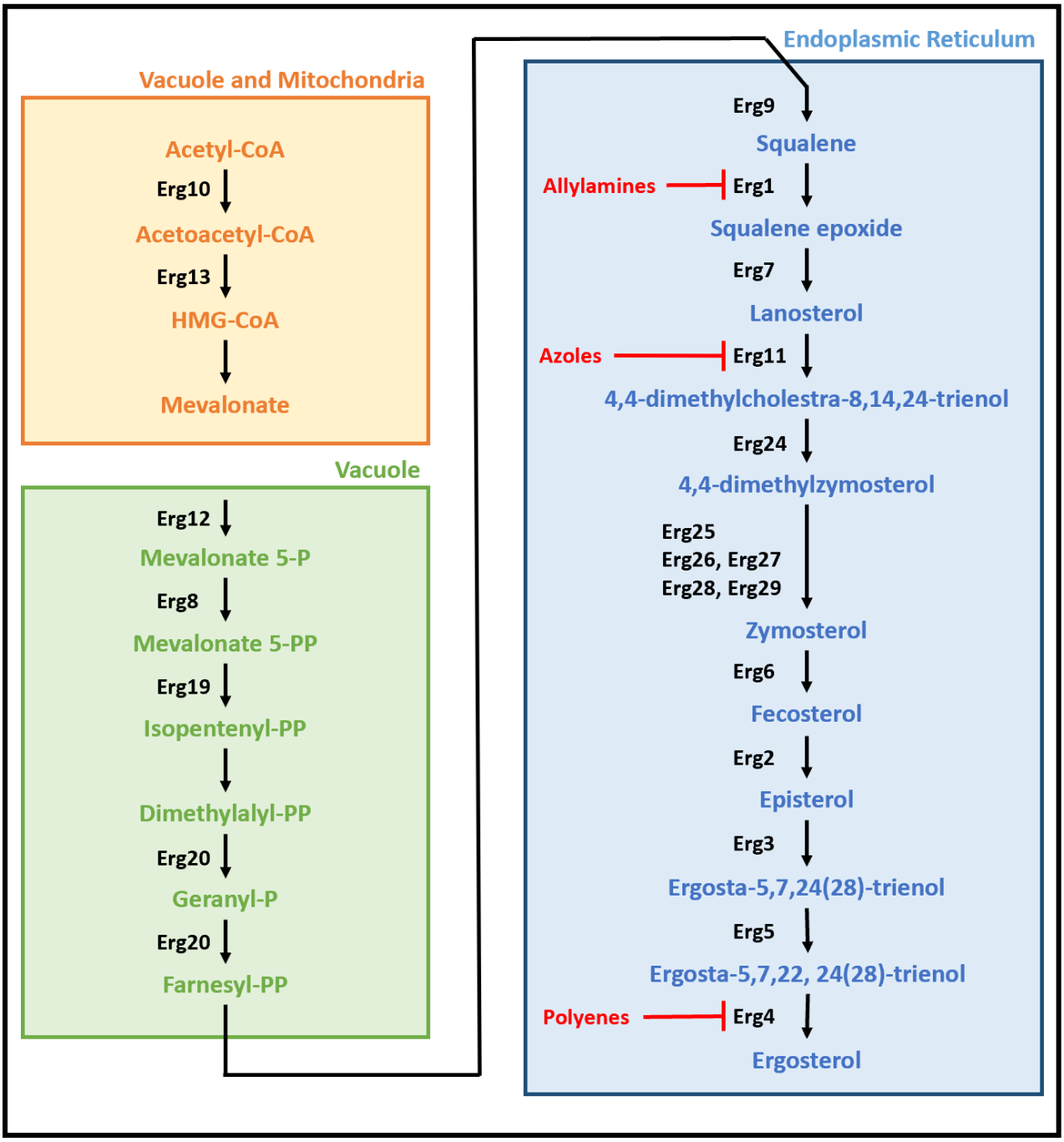

Vacuole and Mitochondria
Acetyl-CoA
Erg10
Acetoacetyl-CoA
Erg13
HMG-CoA
Mevalonate
Vacuole
Erg12
Mevalonate 5-P
Erg8
Mevalonate 5-PP
Erg19
Isopentenyl-PP
Dimethylalyl-PP
Erg20
Geranyl-P
Erg20
Farnesyl-PP
Endoplasmic Reticulum
Erg9
Squalene
Allylamines
Erg1
Squalene epoxide
Erg7
Lanosterol
Azoles
Erg11
4,4-dimethylcholestra-8,14,24-trienol
Erg24
4,4-dimethylzymosterol
Erg25
Erg26, Erg27
Erg28, Erg29
Zymosterol
Erg6
Fecosterol
Erg2
Episterol
Erg3
Ergosta-5,7,24(28)-trienol
Erg5
Ergosta-5,7,22, 24(28)-trienol
Polyenes
Erg4
Ergosterol

Figure 1.5. Ergosterol biosynthetic pathway in *S. cerevisiae*.

The different color boxes represent the three modules of ergosterol synthesis. Orange box: the mevalonate pathway, which occurs in the vacuole and mitochondria. Green Box: farnesyl pyrophosphate (farnesyl-PP) biosynthesis carried out in the vacuole. Blue box: late pathway, ends in ergosterol synthesis, mainly occurs in the ER. (Figure adapted from (Jordá and Puig 2020), and created with BioRender)

1.5 Antifungal drug resistance in pathogenic species

Candida albicans is a normal member of the human epidermal and gastrointestinal microbiome (Fidel 1999). In healthy individuals with a functional immune system, *C. albicans* not only helps with immunity against other pathogens but is continuously sensed by the immune system and maintained at basal levels. However, in immunocompromised individuals, the immune system is unable to maintain the microbiota at basal levels leading to *C. albicans* overgrowth and even life-threatening blood-borne candidiasis (Low and Rotstein 2011; Maertens, Vrebos, and Boogaerts 2001; "Fungal Infections | Fungal | CDC" 2019). There is evidence that activation of the Ire1 pathway and the UPR in common pathogenic fungal species like *C. albicans*, *C. parapsilosis* and *Aspergillus fumigatus*, plays a key role in enabling these species to resist various antifungals (Richie et al. 2009; Wimalasena et al. 2008; Sharma et al. 2019). However, the determinants of this Ire1 dependent pathway remain relatively uncharacterized in pathogenic species, at least partially because robust genome editing tools have only recently become available. Therefore, in this study, I will seek to characterize the importance of the UPR and Ire1 during azole drug resistance through the use of the *S. cerevisiae* model organism.

There are 4 major classes of antifungals available in clinical use today: polyenes, allylamines, azoles, and echinocandins. The first 3 classes compromise the integrity of the cell membrane by attacking the yeast-specific membrane sterol known as ergosterol or inhibiting enzymes responsible for the synthesis of ergosterol. Ergosterol is a cell membrane sterol that serves as a bioregulator of membrane fluidity and consequently is vital for membrane integrity in fungal species (Ghannoum and Rice 1999; Suchodolski et al. 2019). Azoles in particular target the heme protein lanosterol 14 α-demethylase which is responsible for converting lanosterol to ergosterol (Monk et al. 2020). Thus, inhibition of this enzyme leads to the depletion of ergosterol and

accumulation of ergosterol precursors such as 14a-methylated sterols (lanosterol, 4,14-dimethylzymosterol, and 24-methylenedihydrolanosterol), resulting in altered structure and function of the plasma membrane (Ghannoum and Rice 1999). One of the most widely used azoles is fluconazole, which is a fungistatic drug which inhibits growth without destroying the fungal cells and allows the immune system to clear the infection (M. A. Pfaller, Sheehan, and Rex 2004). In contrast, miconazole is a fungicidal drug which works to destroy fungal cells (Sawyer et al. 1975). Echinocandins are a relatively new class of antifungals that compromise the integrity of the cell wall by inhibiting the synthesis of β-glucan by compromising the 1,3-β-glucan synthase enzyme on the cell membrane (Ghannoum and Rice 1999; Lima, Colombo, and de Almeida Junior 2019). Cell wall integrity is essential for the maintenance of turgor pressure and the prevention of cell lysis[23]. Both the cell membrane and cell wall rely heavily on secretory proteins from the ER to maintain their structure and interestingly, perturbation of the cell wall has been associated with UPR activation in yeast (Scrimale et al. 2009). The widespread use of azoles and echinocandins as the primary treatment against candidiasis is therefore associated with the emergence of resistant strains (Brown et al. 2012; Sharma et al. 2019). Indeed, a large proportion of the disease-causing *Candida* isolates display reduced susceptibility to echinocandin and cross-resistance with other antifungals such as azoles, thereby complicating the approach to treatment (M. Pfaller et al. 2012; Garcia-Effron et al. 2010; Alexander et al. 2013). Interestingly, in *C. albicans*, the UPR was shown to be important for polarized growth, and deletion of *IRE1* leads to reduced virulency and hypersensitivity to caspofungin (Blankenship et al. 2010; Wimalasena et al. 2008; Sircaik et al. 2021). The limited antifungal classes available for treatment make it imperative to prolong the use and efficacy of the available drugs and to discover alternative lines of treatment to design more effective therapeutic approaches.

Current literature indicates that, upon treatment with antifungal agents, *IRE1* deletion mutants have significant growth defects in comparison to wild-type strains (Ahmed, Carter, and Lajoie 2019). Therefore, antifungal drug resistance may be conferred due to an essential role played by the upregulation of UPR regulated genes. However, some pathogenic species such as *C. albicans*, and *C. glabrata* display no activation of Hac1-dependent UPR upon treatment with azoles. This suggests that the Ire1 protein may have UPR-independent functions, which is why in this thesis I sought to determine the role of Ire1 in azole resistance through the use of the *S. cerevisiae* model organism.

1.6 *Saccharomyces cerevisiae* as a model organism

Working with pathogenic fungal species can be quite challenging as most of the established tools for genetic manipulation was developed for *S. cerevisiae*. Furthermore, unlike *S. cerevisiae*, species like *C. albicans* and *C. glabrata* have genomes with high levels of plasticity, for example, translocations, truncations, and ploidy are commonly observed (Noble, Gianetti, and Witchley 2017). This makes the utilization of genetic alteration techniques such as homologous recombination and plasmid insertion inefficient in pathogenic species as inserted plasmids and DNA fragments may increase in copy number or may be removed from the genome altogether. In addition, studies have found that experimental conditions of antifungal drug treatment can induce genomic alterations in these pathogenic species (Selmecki, Forche, and Berman 2010). Another advantage of using *S. cerevisiae* as a model organism is due its status as being the most well-studied fungal species. Moreover, the immense amount of existing knowledge which characterizes its genes and pathways are all synthesized into databases such as the gene ontology (GO), *Saccharomyces* genome database (SGD), and TheCellMap, which provides genetic interaction data. Most importantly, since the UPR was first discovered in *S. cerevisiae*, this species has accumulated quite a large repository of information pertaining to the UPR. Therefore, use of this model for my study is warranted as I aim to characterize the function of Ire1 upon treatment with azole drugs.

One limitation of using *S. cerevisiae* as a model in this instance is that its resemblance to pathogenic fungi is relatively limited. Since *S. cerevisiae* is not pathogenic, the essential processes related to growth and virulence are not necessarily conserved with other pathogenic species (Skrzypek et al. 2017; Demuyser and Van Dijck 2019). Additionally, certain aspects of azole susceptibility and genes involved in azole resistance are not shared between pathogenic species

and *S. cerevisiae* (Mount et al. 2018). For example, the primary target of the azoles, Erg11, is an essential gene in *S. cerevisiae*, yet not necessary for viability in *C. albicans* (Bard et al. 1993). However, despite these potential limitations, the use of the *S. cerevisiae* model allows me to characterize fundamental mechanisms of Ire1 in azole resistance easily, and further our understanding of antifungal drug resistance through comparison and extrapolation of discoveries here to more pathogenic species. Furthermore, discoveries from my research can then be explored and adapted for studies using pathogenic species.

Interestingly, several studies link the presence of pathogenic yeast species with gut diseases such as Chron's disease, diarrhea and pouchitis, whereas high levels of *S. cerevisiae* are associated with a healthy gut (Kühbacher et al. 2006; Hoarau et al. 2016; Sangster et al. 2016; Sokol et al. 2017). Therefore, there may be an interest to develop new antifungal drugs which act only against the pathogenic species without harming the commensal *S. cerevisiae* populations of the gut (Demuyser and Van Dijck 2019). As such, my study which specifically studies the effect of azoles on *S. cerevisiae*, makes strides in the ability to differentiate between *S. cerevisiae* and pathogenic species.

1.7 Hypothesis

In *S. cerevisiae*, Ire1 deploys a distinct Hac1-dependent UPR transcriptional program in response to proteotoxic and LBS. However, I postulate that upon azole treatment, Ire1 has a Hac1-independent function which confers azole resistance.

1.8 Objectives

To study my hypothesis, I have 3 main objectives which were all completed with the *S. cerevisiae* model:

Objective 1: Define the requirement for UPR activation during azole resistance

Objective 2: Determine which domains of Ire1 regulates azole resistance

Objective 3: Determine if UPR target genes are required for azole resistance

Chapter 2

2 Materials and Methods

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 W303a. The strains YCR200, YCR201, YCR202, YCR212, YCR213 were obtained from Peter Walter (UCSF) (Rubio et al. 2011). All strains were thawed from frozen stocks and grown on YPD (yeast extract peptone 48 dextrose) or selective SC (synthetic complete) media for 2 days at 30 ℃ before being transferred to liquid cultures. All experiments were carried out using either SC media containing 2 % wv-1 glucose supplemented with 100× inositol or YPD media. Liquid cultures were grown at 30 ℃ in liquid media with constant agitation or on selective agar plates.

Table 2.1: Yeast strains

Strains		Genotype	Reference
W303 Δ*ire1*	YCR200	*ire*Δ*::TRP1, his3::UPRE-LACZ-HIS3*, W303a derivative	(Rubio et al. 2011)
W303 Δ*ire1* + *IRE1*	YCR201	as YCR200, except *ura3::IRE1-3xFLAG-URA3*	(Rubio et al. 2011)
W303 Δ*ire1* + *IRE1* Δ*hac1*	YPL755	as YCR200, except *ura3::IRE1-3xFLAG-URA3, HAC1::KAN*	This study
W303 Δ*ire1* + D797N, K799N	YCR202	as YCR200, except *ura3::ire1(D797N, K799N)-3xFLAG-URA3*	(Rubio et al. 2011)
W303 Δ*ire1* + *IRE1*-GFP	YCR212	as YDP002, except *leu2::IRE1-GFP-LEU2*	(Rubio et al. 2011)
W303 Δ*ire1* + *ire1*(D797N, K799N)-GFP	YCR213	as YDP002, except *leu2::ire1(D797N, K799N)-GFP-LEU2*	(Rubio et al. 2011)
W303 Kar2-ymsfGFP (integrated KAR2 locus)	YPL250	W303a derivative Kar2*::KAR2-ymsfGFP-HIS*	This study
W303 Δ*ire1* + *IRE1* Δ*hrd1*	YPL752	as YCR200, except *ura3::IRE1-3xFLAG-URA3, HAC1::KAN*	This study

2.2 DNA constructs

IRE1 wild type and *ire1*(ΔLD) (luminal domain deletion mutant) gene fragments were digested out of the pRS313 backbone using Not1/Xho1 restriction enzymes and subcloned into the Xho1/Not1 site of pRS41K. The pRS41K vector was used for experiments in order to ensure compatibility with the *ire*Δ*::TRP1, his3::UPRE-LACZ-HIS3* strain.

Table 2.2: Plasmids

Plasmids	Vector Backbone	Resistance marker	Reference
pRS313 *IRE1* WT	pRS313	His	(Ho et al. 2020b)
pRS313 *ire1*(ΔLD)	pRS313	His	(Ho et al. 2020b)
pRS41K *IRE1* WT	pRS41K	G418	This study
pRS41K *ire1*(ΔLD)	pRS41K	G418	This study

2.3 Yeast transformation

Yeast transformations were performed using the lithium acetate protocol described by Gietz and Woods (Gietz and Woods 2002). Briefly, 1 mL of overnight cultures were pelleted and washed with sterile 0.1 M LiAc in TE buffer. Cell pellets were resuspended in 285 µL of 40 % PEG, 10 µL salmon sperm, and 2.5 µL of plasmid (100-500 ng/µl). Cells were then incubated at 30 ˚C with agitation for 45 minutes. Next, 45 µl of DMSO was added and yeast were heat-shocked for 15 mins at 42 ˚C in a water bath before being plated on amino acid or chemical selection plates.

2.4 Yeast genetic manipulation

Gene deletions were performed by synthesizing a DNA fragment containing the 5' and 3' untranslated regions (UTR) of the *HAC1* and *HRD1* genes and the protein coding sequence of the resistance marker for G418 (*KAN*). Then, the PCR products were transformed into the *Δire1 + IRE1* strain to allow the 5' and 3' UTRs of the fragments to recombine into their respective locus on the yeast genome through homologous recombination.

2.5 Drugs

Stock solutions of tunicamycin (5 mg/mL in DMSO; Amresco), dithiothreitol (DTT) (1 M in DMSO; Promega), fluconazole (50 mg/mL in DMSO; Alfa Aesar), and miconazole (50 µg/mL

in DMSO; Alfa Aesar) were used at the indicated concentrations. A stock of 250 µg/mL Amphotericin B (Gibco) was used at the indicated concentrations.

2.6 Spotting assays

Cell growth was assessed by spot assays following the protocol described by Petropavlovskiy et al. (Petropavlovskiy et al. 2020). Briefly, yeast cells were cultured overnight in either YPD containing the appropriate selection drug, or 2 % glucose synthetic selective media without the appropriate selection amino acid. OD_{600} of overnight cultures was measured and diluted to OD_{600} 0.1 in 96 well plates. Then five 1:5 sequential serial dilutions were performed by pipetting 40 µL from the previous column to 160 µL of YPD in the adjacent column. Equal volumes of the cultures were transferred to dry chemical plates (0.3 µg/mL tunicamycin, 15-40 µg/mL fluconazole, and 0.02-0.05 µg/ml miconazole) using a 48-prong spotter. Plates were grown at 30 ˚C for 2-3 days and imaged using a mounted camera. Relative growth values were generated by measuring the grey values using ImageJ software. Then, the average grey values of each strain in the treated conditions were determined relative to the untreated and tunicamycin treated conditions.

2.7 *HAC1* splicing assay

Liquid cultures of W303 Δ*ire1*, Δ*ire1* + *IRE1*, Δ*ire1* + *ire1*(D797N, K799N) cells were grown overnight in -His SC media. Cultures were diluted to 0.1 OD_{600} in 50 mL of new -His SC media and grown for 4 h at 30 ˚C with agitation to reach early log phase. 5 mL aliquots of the cultures were treated with the indicated drugs in culture tubes and placed back at 30 ˚C in a culture rotator for indicated times. Then, cells were harvested by centrifugation and RNA extractions were performed using the MasterPure Yeast RNA Purification Kit (Epicentre). *HAC1* cDNA was synthesized from the extracted RNA using Superscript IV One-Step RT-PCR System Protocol

with *HAC1* forward and reverse primers (listed in Table 2.3). Resulting PCR products were separated by electrophoresis on a 1 % agarose gel supplemented with 10 % ethidium bromide. Bands were visualized using the Bio-Rad Gel Doc system (Bio-Rad). The spliced % was calculated by dividing the $HAC1^s$ grey value by the sum of $HAC1^s$ and $HAC1^u$ grey values measured through ImageJ.

Table 2.3: Primers used for *HAC1* splicing assay and RT-PCR.

Gene	Forward Primer	Reverse Primer
HAC1	ACGACGCTTTTGTTGCTTCT	TCTTCGGTTGAAGTAGCACAC
ACT1	CACCCTGTTCTTTTGACTGA	CGTAGAAGGCTGGAACGTTG
ERG25	TTTCTTGGTCGAGGCCATCC	CCGAATGGAGCAGCGTATCT
ERG11	TGCACCATCCATTGCACTCT	CCGACGGAATAAGAGGAGGC
ERG7	TCAAGGCCCCACTAGCAATG	TGTCAACCCAGAACGGATG

2.8 Fluorescence Microscopy

Early log phase cells were cultured and treated as described above for the *HAC1* splicing assay. Confocal images were obtained on a Zeiss LSM 800 AiryScan confocal microscope equipped with 488 nm and 561 nm diode lasers and a 63x PlanApochromat NA 1.4 objective, located within the Anatomy and Cell Biology Imaging Core Facility. Images were analyzed using ImageJ software.

2.9 β-galactosidase Assay

W303 Δ*ire1* and Δ*ire1* + *IRE1* carrying the *UPRE*-LacZ reporter were used. OD_{600} of overnight cultures were measured and diluted to OD_{600} 0.2 in 50 mL of new 2 % glucose -His SC media and grown for 4 h at 30 ˚C with continuous agitation to reach early log phase. Cultures were

treated with indicated drugs in aliquots of 5 mL and placed at 30 ˚C in culture rotator for 4 h. Cells were harvested by centrifugation and resuspended in *LacZ* buffer. To measure β-galactosidase activity, 50 mL cell lysate was mixed with 950 mL *LacZ* buffer containing 2.7 mL β-mercaptoethanol, 1 drop (50 µL) 0.1 % SDS (Sodium dodecyl sulfate), 2 drops (100 µL) $CHCl_3$ and incubated at 30 ˚C for 15 min. The reaction was started by adding 100 mL ONPG (ortho-Nitrophenyl-ß-galactoside) (4 mg/mL) and incubated at 30 ˚C until the colour changed to yellow. The reaction was stopped by adding 300 mL of 1 M Na_2CO_3. β-galactosidase activity was determined at 420 nm absorbance, normalizing data to cell density determined at 600 nm absorbance. Data was analysed and graphed using GraphPad (Prism).

2.10 Flow cytometry

Early log phase W303 Kar2-ymsfGFP cells were cultured and treated as described above for the *HAC1* splicing assay. Quantification of cells expressing fluorescent reporters was performed using the BD Bioscience FACS Celesta flow cytometer equipped with a 488 nm laser for imaging GFP. Data were analyzed using the Flowing Software (Turku Bioscience Centre https://bioscience.fi/services/cell-imaging/flowing-software/). All conditions were performed in triplicate, 10,000 cells were analyzed per sample, and median fluorescence intensities were used for analysis and graphed using GraphPad (Prism). No gates were applied.

2.11 Quantitative RT-PCR

RNA extractions were performed using the MasterPure Yeast RNA Purification Kit (Epicentre). cDNA was synthesized from the extracted RNA using the Superscript IV VILO Master Mix Protocol. The cDNA preparations were used as templates for amplification using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) using the primers listed in Table 2.3.

The relative expression levels were analyzed using the comparative ΔΔCt method with *ACT1* as a housekeeping gene for normalization and graphed using GraphPad (Prism).

2.12 Statistical analysis

For all experiments, ordinary one-way ANOVA followed by the Šidák multiple comparison test was used to determine statistical significance of experimental conditions using GraphPad (Prism). All graphs are scatter dot plots with line at mean with SD. ***$P < 0.0001$, ***$P = 0.0001$, **$P = 0.0017$, ns $P > 0.9999$. All experiments were done in triplicates, ($n = 3$).

Chapter 3

3 Results

3.1 Ire1 regulates azole resistance independently of Hac1

The Ire1 protein is directly responsible for the detection of ER stress and indirectly for the regulation of genes associated with the UPR (Sidrauski and Walter 1997; Mori et al. 1996). Previous studies have established that *IRE1* is a vital gene for proteotoxic stress mediation (Cox, Shamu, and Walter 1993). To determine if the presence of the Ire1 protein is important for azole resistance, I employed an *IRE1* deletion (Δ*ire1*) mutant in the W303a background strain and treated the Δ*ire1* strain with the proteotoxic stressor tunicamycin (0.3 µg/mL), an N-glycosylation inhibitor and canonical ER stressor. As expected, the Δ*ire1* strain showed no growth upon treatment with tunicamycin, while the same strain complemented with an integrated *IRE1* plasmid showed no observable growth defect (Figure 3.1 A). Moreover, unlike the *IRE1* complemented strain, the Δ*ire1* mutant showed a significant growth defect in the presence of the azole antifungals fluconazole (15 µg/mL) and miconazole (0.02 µg/mL) (Figure 3.1 A, B). These findings suggest that the presence of the Ire1 protein is important for azole drug resistance.

Next, to determine if the UPR is activated in response to treatment with azoles, I deleted the *HAC1* gene in the *IRE1* complemented strain. Only spliced *HAC1* mRNA can be translated into the Hac1 transcription factor responsible for UPR gene regulation (Cox and Walter 1996; Chapman and Walter 1997; Sidrauski, Chapman, and Walter 1998). Thus, deletion of the *HAC1* gene prevents UPR activation altogether. Prior research has established that the *HAC1* gene is vital for proteotoxic stress mediation (Cox, Chapman, and Walter 1997). As expected, Δ*hac1* showed no growth upon treatment with tunicamycin, supporting the notion that loss of the *HAC1* gene is detrimental for yeast growth following exposure to tunicamycin (Figure 3.1 A). Upon treatment with fluconazole and miconazole, the Δ*hac1* strain showed an identical growth phenotype to the *IRE1* complemented strain, indicating that Hac1 is dispensable for growth in the presence of azoles

(Figure 3.1 A, B). To achieve statistical significance, one more replicate is required. Overall, this suggests that the UPR is not required for tolerance of azole treatment.

To validate that UPR activation via splicing of the *HAC1* is truly dispensable for azole tolerance, we performed a *HAC1* splicing assay on the *IRE1* complemented strain. *HAC1* mRNA splicing assays assess the level of splicing and indirectly indicate the level of UPR activity. As expected, when the *IRE1* complemented strain was treated with tunicamycin, 41.2 % *HAC1* mRNA (*HAC1*^{s}) was spliced (Figure 3.1 C). However, when treated with azoles the percent of *HAC1* spliced was comparable to the untreated condition, further suggesting that the UPR is not activated in response to azole treatment (Figure 3.1 C). Azole concentrations of 40 µg/mL fluconazole and 0.05 µg/mL miconazole were used to validate that not even higher concentrations result in *HAC1* splicing.

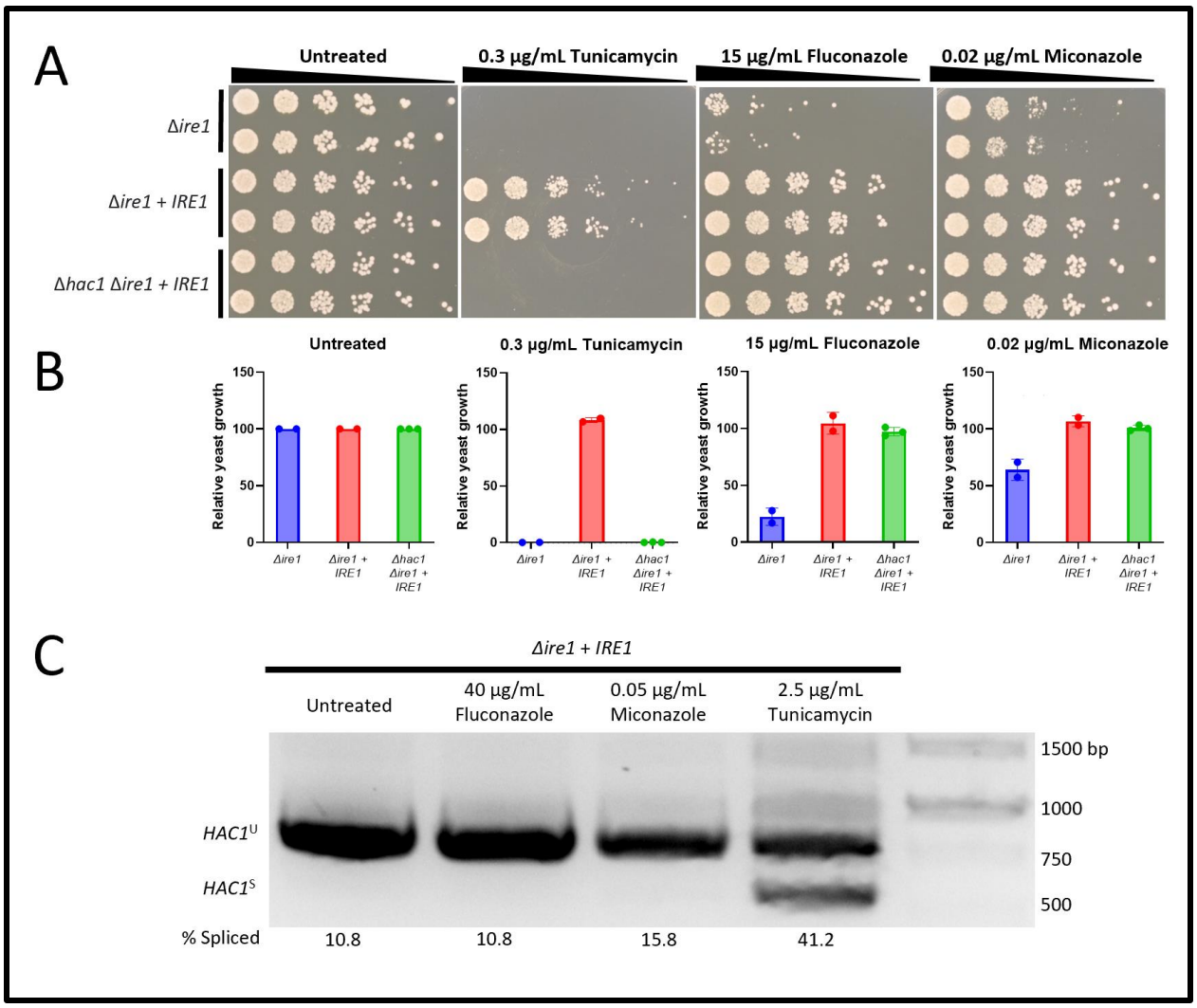

A
Untreated
0.3 µg/mL Tunicamycin
15 µg/mL Fluconazole
0.02 µg/mL Miconazole
Δire1
Δire1 + IRE1
Δhac1 Δire1 + IRE1
B
Untreated
0.3 µg/mL Tunicamycin
15 µg/mL Fluconazole
0.02 µg/mL Miconazole
Relative yeast growth
150
100
50
0
Δire1
Δire1 + IRE1
Δhac1 Δire1 + IRE1
C
Δire1 + IRE1
Untreated
40 µg/mL Fluconazole
0.05 µg/mL Miconazole
2.5 µg/mL Tunicamycin
1500 bp
1000
750
500
HAC1U
HAC1S
% Spliced
10.8
10.8
15.8
41.2

Figure 3.1: Azole tolerance requires Ire1 through a Hac1-independent mechanism.

(A) For phenotypic comparison, fivefold serial dilutions of 0.1 OD_{600} cell suspensions of Δ*ire1*, Δ*ire1 + IRE1* and Δ*hac1* Δ*ire1 + IRE1* were spotted onto YPD plates supplemented with 15 µg/mL fluconazole, 0.02 µg/mL miconazole, and 0.3 µg/mL tunicamycin. Growth of strains on the untreated plate were used as controls. Plates were incubated at 30 ˚C for 2 days and imaged using a mounted camera.

(B) Quantification of growth plates shown in A, graphed using GraphPad (Prism). Gray values of the 5× dilution of each strain in all plates were measured with ImageJ. Relative growth was calculated and graphed in a scatter dot plot with line at mean with SD, n = 3. ***$P < 0.0001$, ns $P > 0.9999$

(C) Analysis of *HAC1* splicing in the Δ*ire1 + Ire1* strain post 2 h treatment with 40 µg/mL fluconazole, 0.05 µg/mL miconazole and 2.5 µg/mL tunicamycin. Percent spliced was calculated by dividing the $HAC1^s$ grey value by the sum of $HAC1^s$ and $HAC1^u$ grey values measured through ImageJ.

I next determined if transcription from Hac1-driven promoters occur during azole treatment. I performed a β-galactosidase assay with the *IRE1* complemented strain using a *LacZ* reporter gene under the control of the *UPRE*. As expected, when treated with tunicamycin, there was significant increase in β-galactosidase production. Fluconazole was used at 20 μg/mL in this experiment to determine UPR activation at higher azole concentrations. In line with our previous findings, there was no increase in β-galactosidase production during treatment with 20 μg/ml fluconazole when compared to the untreated condition (Figure 3.2), indicating that the *UPRE* controlling the expression of the LacZ reporter gene is not upregulated upon treatment with azoles. We therefore conclude that the Ire1 protein is required for azole resistance through a UPR-independent mechanism. To the best of our knowledge, this is the first instance of a UPR independent function of Ire1 reported in *S. cerevisiae*.

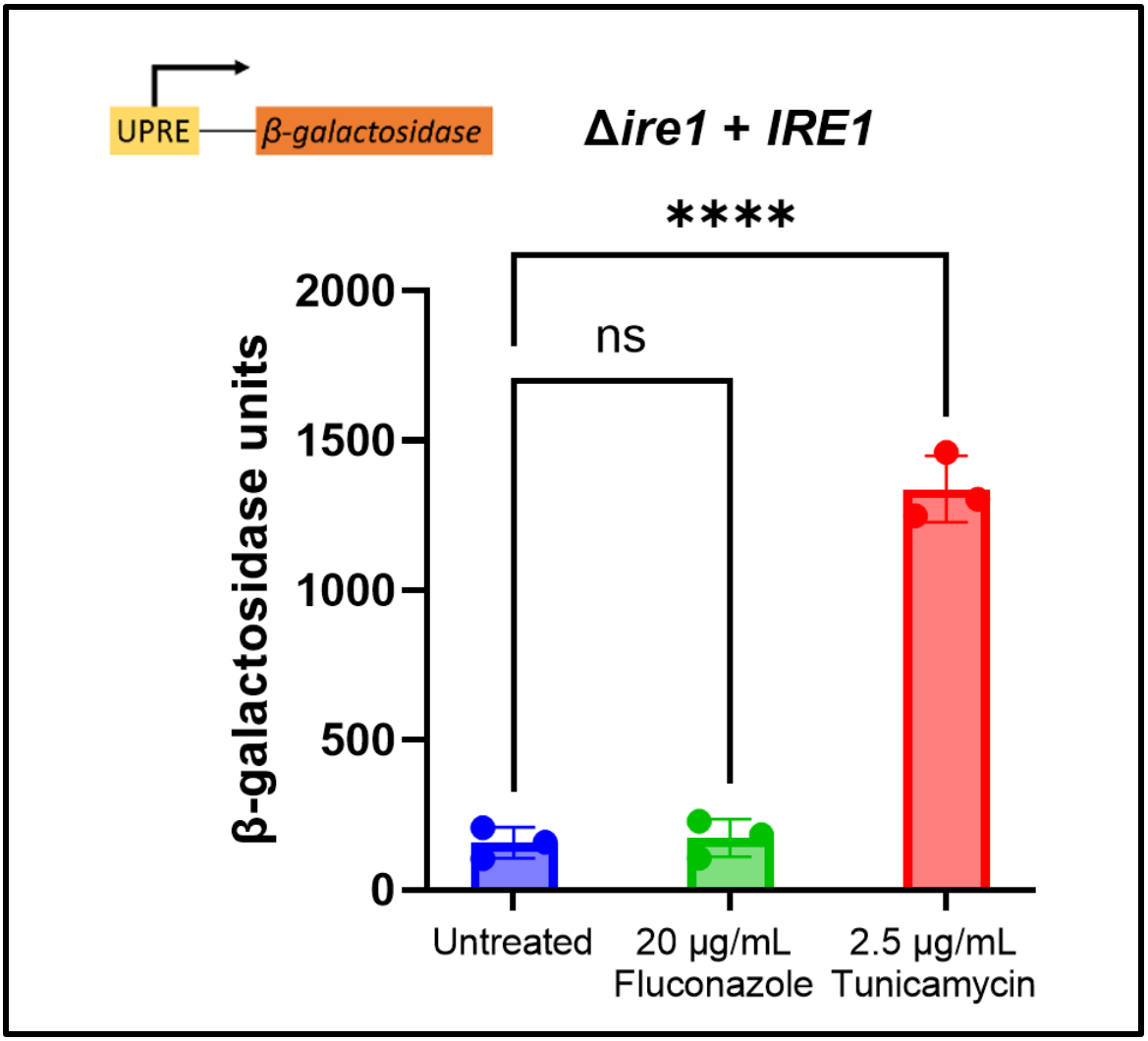

UPRE
β-galactosidase
Δire1 + IRE1
****
ns
2000
1500
1000
500
0
β-galactosidase units
Untreated
20 µg/mL Fluconazole
2.5 µg/mL Tunicamycin

Figure 3.2: Azole treatment does not induce activation of the *UPRE* promoter

Early log phase culture of Δ*ire1* + *IRE1* was treated with 20 µg/mL fluconazole, and 2.5 µg/mL tunicamycin for 3 h. β-galactosidase units were measured using A_{420} (for fluorescence), A_{600} (for cell density) values and the length of time allowed for reaction with ONPG. Graph is a scatter dot plot with line at mean with SD, made with GraphPad (Prism). Experiment was conducted in triplicate. ****$P < 0.0001$, ns $P > 0.9999$

Currently, the proposed theory of Ire1 activation is that, upon ER stress such as proteotoxic and LBS, Ire1 monomers form dimers which fuse to become large clusters during the first hours of ER stress (Aragón et al. 2009; Kimata et al. 2007; van Anken et al. 2014). Once the ER stress is resolved or if the ER stress is sustained, the Ire1 clusters are dissolved back into monomers. The Ire1 cluster assembly is closely associated with *HAC1* splicing and UPR activation, while the dissolution of the clusters results in termination of *HAC1* splicing and UPR attenuation (van Anken et al. 2014; Rubio et al. 2011). To determine if azole treatment induces Ire1 clustering, we employed a strain in which Ire1 was tagged with GFP. As expected, upon treatment with tunicamycin, Ire1-GFP was observed to cluster (red arrows in figure 3.3). However, we were unable to observe significant clustering upon treatment with fluconazole and miconazole (Figure 3.3). This indicates that azoles do not result in the canonical activation of Ire1. Furthermore, these findings indirectly suggest that azoles do not result in proteotoxic or LBS, and thus supports the previous findings of absent *HAC1* splicing and subsequent UPR activation upon treatment with azoles.

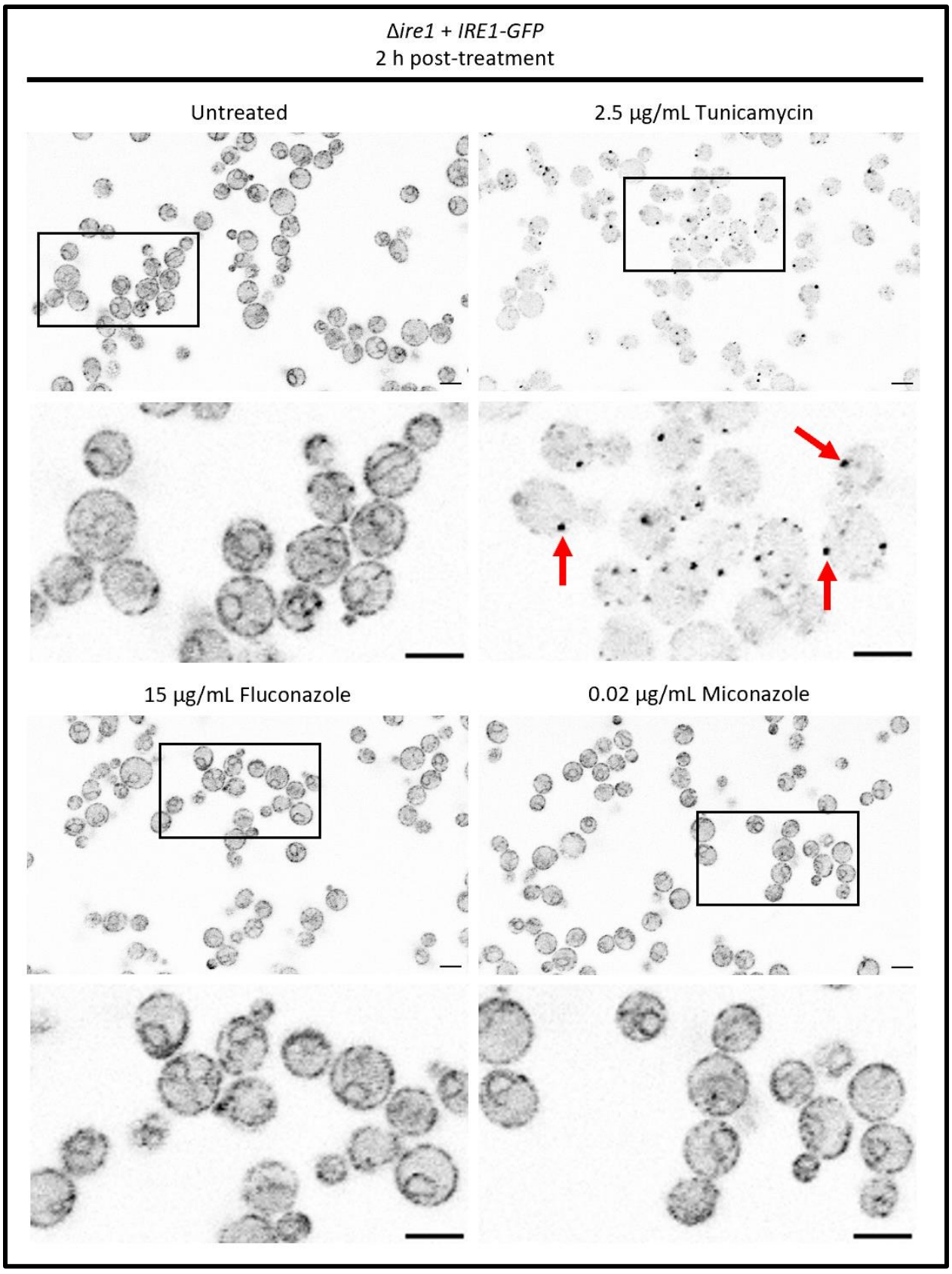

Δire1 + IRE1-GFP
2 h post-treatment
Untreated
2.5 µg/mL Tunicamycin
15 µg/mL Fluconazole
0.02 µg/mL Miconazole

Figure 3.3: Azole treatment does not induce Ire1 clustering

Aliquots of the early log phase Δ*ire1 + IRE1* culture was treated with 2.5 µg/mL tunicamycin, 15 µg/mL fluconazole, and 0.02 µg/mL miconazole for 2 h and imaged using confocal microscopy. Red arrows point to the Ire1-GFP clusters. Scale bars represent a length of 5 µm.

3.2 The Ire1 kinase domain and luminal domain are dispensable for azole resistance.

The residues D797 and K799 coordinate the terminal phosphate of ATP bound to the Ire1 kinase and catalyzes phosphotransfer (Rubio et al. 2011). By mutating these residues to asparagine, the Ire1 kinase activity is abolished without affecting RNase activity, overall steric packaging, hydrophobicity, and hydrogen bonding at the kinase active site. It was found that upon treatment with dithiothreitol (DTT), the *ire1*(D797N, K799N) mutant was able to form oligomers like that of the *IRE1* complemented strain. However, unlike the *IRE1* complemented strain, the *ire1*(D797N, K799N) mutant was unable to dissociate these clusters upon proteotoxic stress adaptation. Instead, the mutant strain continued to increase clustering and UPR activation, thereby leading to impaired cell growth. Therefore, the *ire1*(D797N, K799N) mutant fails to adapt to prolonged ER stress due to an inability to attenuate the UPR despite normal induction of UPR targets.

To determine if the kinase domain is required for azole resistance, I performed spotting assays using the *ire1*(D797N, K799N) mutant strain. As expected, upon treatment with tunicamycin, the growth of the *ire1*(D797N, K799N) mutant was significantly impaired compared to the *IRE1* complemented strain (Figure 2.4 A, B). Interestingly, upon treatment with the azole antifungals fluconazole and miconazole, the *ire1*(D797N, K799N) mutant strain had a similar growth phenotype to the *IRE1* complemented strain. This result indicated that the kinase domain functionality of Ire1 is dispensable during growth in the presence of azoles. We also conducted *HAC1* splicing assays with the *ire1*(D797N, K799N) mutant, and found no observable splicing post treatment with azoles (Figure 2.4 C). These findings further support the conclusion that azole treatment does not activate the UPR, as the inability to attenuate the UPR is dispensable for tolerance of azole treatment.

To investigate the clustering activity of *ire1*(D797N, K799N) upon azole and tunicamycin treatment, we utilized *ire1*(D797N, K799N)-GFP. Interestingly, we found that treatment with 0.3 µg/mL tunicamycin induced the clustering of the Ire1(D797N, K799N)-GFP mutant (red arrows in figure 3.4 D), unlike that of the Ire1-GFP (supplemental figure 1). This indicates that the kinase mutant is more sensitive to proteotoxic stress caused by tunicamycin. Moreover, similar to Ire1-GFP (Figure 3.3), we found that the *ire1*(D797N, K799N)-GFP did not display any clustering upon treatment with azoles (Figure 3.4 D), thereby indicating that the loss of kinase activity does not change the clustering activity upon treatment with azoles. Taken together, these findings suggest that upon azole treatment, the function of the *ire1*(D797N, K799N) mutant with an inactive kinase domain is not distinct from the wild type Ire1, thereby indicating that a functional kinase domain is not required for azole resistance.

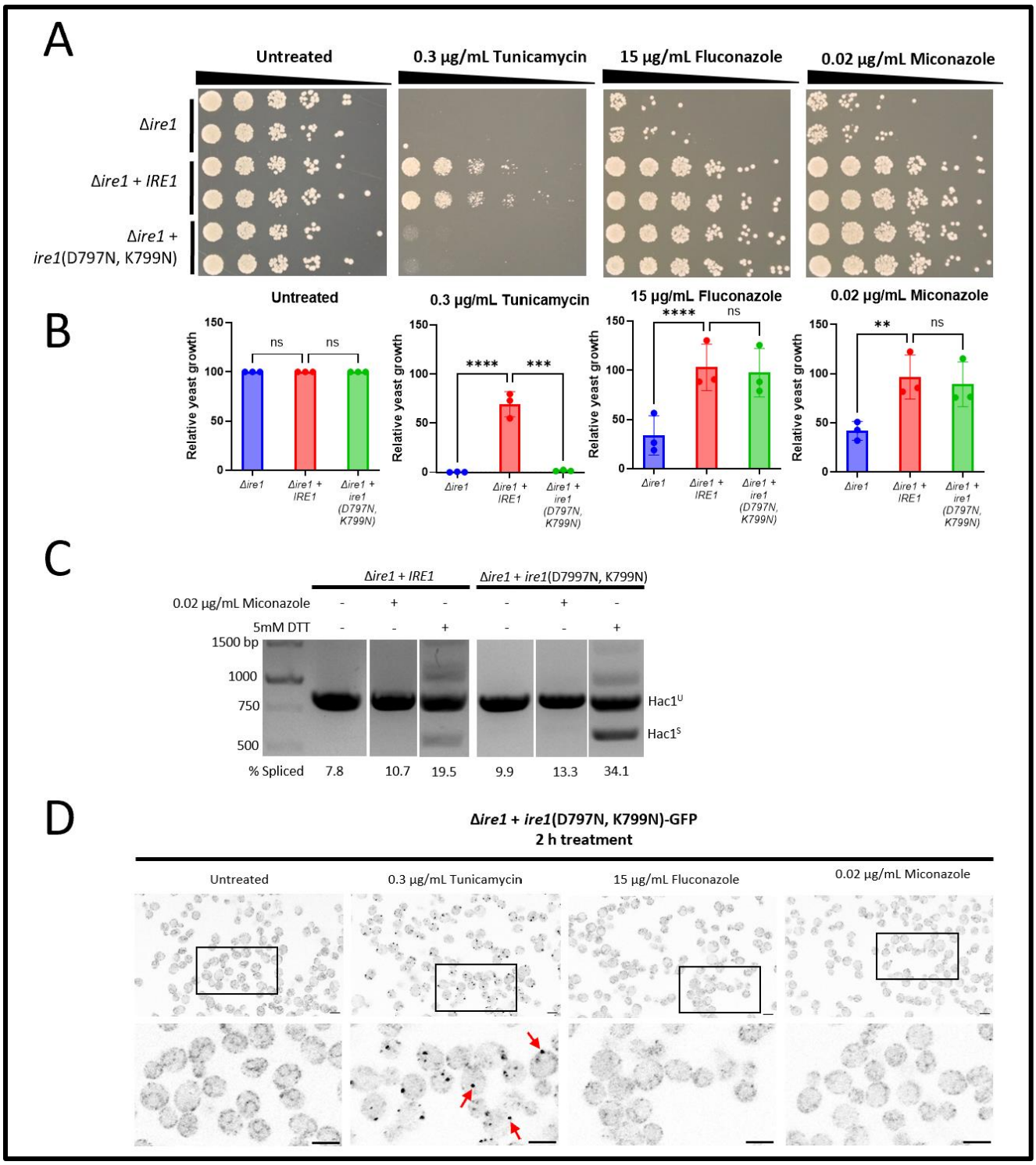

A
Untreated
0.3 µg/mL Tunicamycin
15 µg/mL Fluconazole
0.02 µg/mL Miconazole
Δire1
Δire1 + IRE1
Δire1 + ire1(D797N, K799N)
B
Untreated
0.3 µg/mL Tunicamycin
15 µg/mL Fluconazole
0.02 µg/mL Miconazole
Relative yeast growth
ns
****
***
**
Δire1
Δire1 + IRE1
Δire1 + ire1 (D797N, K799N)
C
Δire1 + IRE1
Δire1 + ire1(D7997N, K799N)
0.02 µg/mL Miconazole
5mM DTT
1500 bp
1000
750
500
Hac1U
Hac1S
% Spliced
7.8
10.7
19.5
9.9
13.3
34.1
D
Δire1 + ire1(D797N, K799N)-GFP
2 h treatment
Untreated
0.3 µg/mL Tunicamycin
15 µg/mL Fluconazole
0.02 µg/mL Miconazole

Figure 3.4: Azole tolerance does not require the kinase domain of Ire1.

(A) For phenotypic comparison, fivefold serial dilutions of 0.1 OD_{600} cell suspensions of Δ*ire1*, Δ*ire1* + *IRE1* and Δ*ire1* + *ire1*(D797N, K799N) were spotted onto YPD plates supplemented with 0.3 μg/mL tunicamycin, 15 μg/mL fluconazole, and 0.02 μg/mL miconazole. Growth of strains on the untreated plate were used as controls. Plates were incubated at 30 ˚C for 2 days and imaged using a mounted camera.

(B) Quantification of growth plates shown in A. Gray values of the 5× dilution of each strain in all plates were measured with ImageJ. Relative growth was calculated and graphed in a scatter dot plot with line at mean with SD, n = 3. ***$P < 0.0001$, ***$P = 0.0001$, **$P = 0.0017$, ns $P > 0.9999$

(C) Analysis of *HAC1* splicing in the Δ*ire1* + *Ire1* and Δ*ire1* + *ire1*(D797N, K799N) strains post 2 h treatment with 0.02 μg/mL miconazole, and 5 mM DTT. Percent spliced was calculated by dividing the $HAC1^s$ grey value by the sum of $HAC1^s$ and $HAC1^u$ grey values measured through ImageJ.

(D) Overnight culture was diluted to 0.1 OD_{600} in 50 mL of new -Leu selective media and incubated at 30 ˚C for 4 h. Then 5 mL aliquots of the culture were treated with stated antifungals for 2 h and imaged using confocal microscopy. Red arrows point to the ire1(D797N, K799N)-GFP clusters. Scale bars represent a length of 5 μm.

As the canonical trigger for Ire1 activation is misfolded protein accumulation in the ER lumen (Ho et al. 2020b), we next determined if proteotoxic stress detection through the luminal domain of Ire1 is required for growth in the presence of azoles. Previous research has shown that the luminal domain is indispensable for the detection of proteotoxic stress, therefore we deleted the luminal domain of *IRE1* (Ho et al. 2020b). The mutant lacking the luminal domain termed *ire1*(ΔLD) remains capable of activating the UPR through LBS detection (Ho et al. 2020b). As expected, when treated with tunicamycin, the *ire1*(ΔLD) mutant had severe growth impairments (Figure 3.5). Interestingly, when treated with azoles, the *ire1*(ΔLD) mutant had a growth phenotype similar to that of the *IRE1* complemented strain. The growth defect displayed by the strains in the fluconazole treated plate is likely due to an interaction between G418, a constituent of the plate used for selective purposes, and fluconazole. Nevertheless, the difference in growth between the *ire1*(ΔLD) and Δ*ire1* + *IRE1* is not significant. In conclusion, these results indicate that the luminal domain is not required for growth in the presence of azoles.

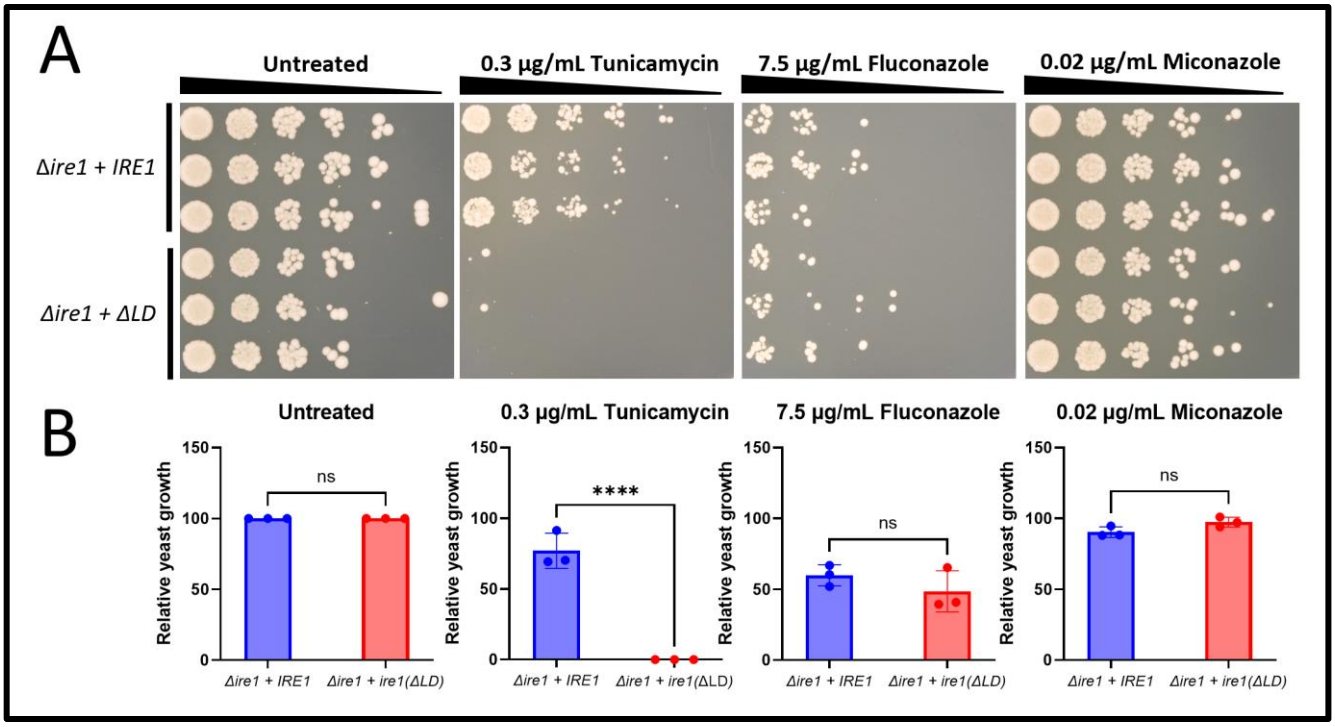

A
Untreated
0.3 µg/mL Tunicamycin
7.5 µg/mL Fluconazole
0.02 µg/mL Miconazole
Δire1 + IRE1
Δire1 + ΔLD
B
Untreated
0.3 µg/mL Tunicamycin
7.5 µg/mL Fluconazole
0.02 µg/mL Miconazole
Relative yeast growth
150
100
50
0
ns
****
ns
ns
Δire1 + IRE1
Δire1 + ire1(ΔLD)

Figure 3.5: Proteotoxic stress detection is not required for growth following exposure to azoles.

(A) For phenotypic comparison, fivefold serial dilutions of 0.1 OD_{600} cell suspensions of Δ*ire1* + *IRE1* and Δ*ire1* + *ire1*(ΔLD) were spotted onto YPD plates supplemented with 0.3 μg/mL tunicamycin, 7.5 μg/mL fluconazole, and 0.02 μg/mL miconazole. Growth of strains on the untreated plate were used as controls. All plates were also treated with 100 μg/mL of the selection marker G418. Plates were incubated at 30 ˚C for 2 days and imaged using a mounted camera.

(B) Quantification of growth plates shown in A. Gray values of the 5× dilution of each strain in all plates were measured with ImageJ. Relative growth was calculated and graphed in a scatter dot plot with line at mean with SD, n = 3. ***$P < 0.0001$, ns $P > 0.9999$

3.3 The UPR targets, Kar2 and Hrd1, are not required for azole resistance.

To reduce the risk of detrimental protein misfolding within the secretory pathway, cells deploy the quality control mechanism ERAD to detect and eliminate misfolded proteins (Araki and Nagata 2011). ERAD is mediated by a set of proteins which includes Kar2, an Ire1 associated essential chaperone protein which dissociates from Ire1 upon binding to misfolded proteins (Kim, Spear, and Ng 2005, 9; Mehnert et al. 2015). Kar2 functions by aggregating with misfolded proteins to mediate correct protein folding within the ER lumen. The dissociation of Kar2 from Ire1 is hypothesized to promote Ire1 clustering and UPR activation (Read and Schröder 2021; Armstrong et al. 2017; Lee et al. 2008; Shamu and Walter 1996). Therefore, unsurprisingly, the *KAR2* gene is regulated by the *UPRE* (Mori et al. 1992; Ho et al. 2020b). To validate that *KAR2* is not upregulated upon treatment with azoles, we performed flow cytometry of GFP tagged Kar2 (Figure 3.6 A). As expected, when treated with tunicamycin, the mean fluorescence intensity of the *KAR2*-GFP was significantly higher than the untreated condition, thereby validating that upon the induction of canonical ER stress, Kar2-GFP synthesis is increased. Furthermore, in line with our prior findings, Kar2-GFP levels following azole treatment were comparable to untreated cells. This suggests that *KAR2* upregulation is dispensable for azole resistance, and that ERAD-L and M are likely not required. Moreover, as Kar2 is known to be upregulated upon detection of misfolded proteins (Mori et al. 1992; Ho et al. 2020b), this result also validates my previous observations; azoles do not culminate in proteotoxic stress.

In addition to Kar2, ERAD functions through a set of proteins which work together to recognize, retrotranslocate and degrade misfolded proteins (Vembar and Brodsky 2008). Hrd1 in particular, is vital for the retro-translocation of misfolded proteins from the ER lumen into the cytosol in the ERAD-L and M pathways (Ye et al. 2004; Lilley and Ploegh 2004). Interestingly,

deletion of *HRD1* induces LBS suggesting a link between ERAD and lipid homeostasis. Therefore, we next investigated the requirement of Hrd1 for tolerance of azole treatment. As expected, deletion of the *HRD1* gene (Δ*hrd1*) resulted in the inability to grow when treated with tunicamycin. However, the Δ*hrd1* mutants had a similar growth phenotype to the *IRE1* complemented strain following exposure to fluconazole and miconazole (Figure 3.6 B and C). To achieve statistical significance, 2 more replicates are required. This result further indicates that Hrd1 dependent ERAD-L and M are not required for azole tolerance and indirectly supports my previous finding that azole tolerance does not require misfolded protein degradation in both the ER lumen and membrane.

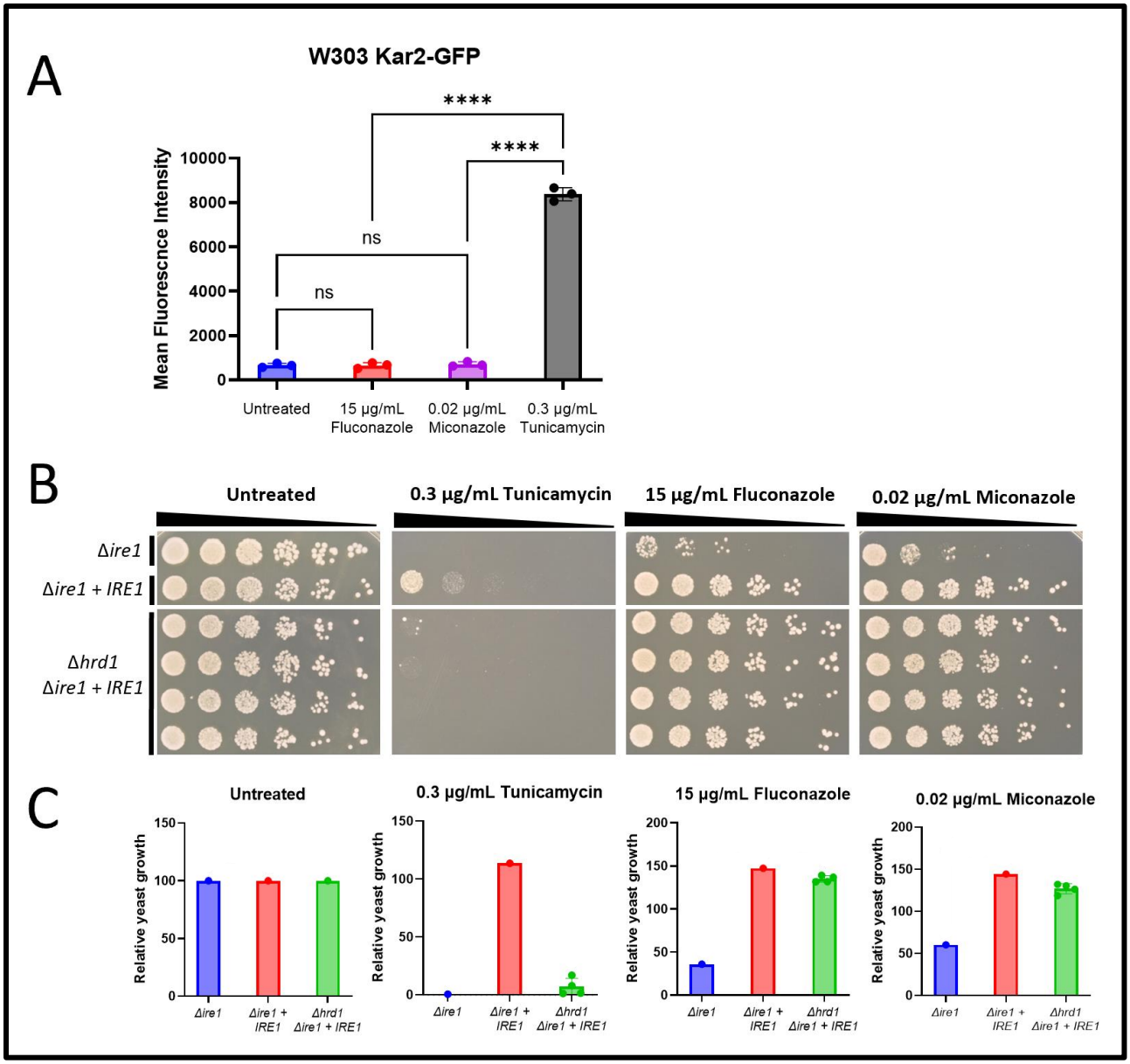

A
W303 Kar2-GFP
Mean Fluorescnce Intensity
10000
8000
6000
4000
2000
0
****
****
ns
ns
Untreated
15 µg/mL Fluconazole
0.02 µg/mL Miconazole
0.3 µg/mL Tunicamycin
B
Untreated
0.3 µg/mL Tunicamycin
15 µg/mL Fluconazole
0.02 µg/mL Miconazole
Δire1
Δire1 + IRE1
Δhrd1
Δire1 + IRE1
C
Untreated
0.3 µg/mL Tunicamycin
15 µg/mL Fluconazole
0.02 µg/mL Miconazole
Relative yeast growth
Δire1
Δire1 + IRE1
Δhrd1 Δire1 + IRE1

Figure 3.6: Azole tolerance does not require the ERAD.

(A) Aliquots of the early log phase W303 *KAR2*-GFP culture was treated with 15 µg/mL fluconazole, 0.02 µg/mL miconazole, and 0.3 µg/mL tunicamycin for 4 h. Fluorescence was quantified using FACS Celesta flow cytometer. Data was analyzed using Flowing Software (Turku Bioscience Centre) and graphed using Prism (Graphpad). Graph is a scatter dot plot with line at mean with SD, n = 3. ***$P < 0.0001$, ns $P > 0.9999$

(B) For phenotypic comparison, fivefold serial dilutions of 0.1 OD_{600} cell suspensions of Δ*ire1*, Δ*ire1* + *IRE1* and Δ*hrd1* Δ*ire1* + *IRE1* were spotted onto YPD plates supplemented with 0.3 µg/mL tunicamycin, 15 µg/mL fluconazole, and 0.02 µg/mL miconazole. Growth of strains on the untreated plate were used as controls. Plates were incubated at 30 ˚C for 2 days and imaged using a mounted camera.

(C) Quantification of growth plates shown in B. Gray values of the 5× dilution of each strain in all plates were measured with ImageJ. Relative growth was calculated and graphed in a scatter dot plot with line at mean with SD, n = 3. ***$P < 0.0001$, ns $P > 0.9999$

3.4 Ergosterol synthesis genes are downregulated in the absence of Ire1.

Genome wide transcriptional profiling data of *C. albicans* done by Sircaik et al., showed that in the *IRE1*-DX (diminished expression) strain, ergosterol synthesis genes were significantly downregulated compared to the wild type (Sircaik et al. 2021). This prompted us to look at the expression levels of ergosterol synthesis genes in *S. cerevisiae*. Similarly, my preliminary qRT-PCR results indicate that ergosterol synthesis genes, specifically *ERG25, ERG11,* and *ERG7* in the Δ*ire1* strain of *S. cerevisiae* are downregulated, when compared to the *IRE1* complemented strain (Figure 3.7 A). However, this experiment should be replicated to establish statistical significance. Importantly, Erg11 has been shown to be the rate limiting step of ergosterol synthesis (reviewed in (Hu et al. 2017; Jordá and Puig 2020)). Therefore, its downregulated expression strongly suggests changes in the levels of ergosterol.

We also treated the Δ*ire1* cells with amphotericin B, a drug that directly binds to ergosterol and forms pores in the cell membrane, leading to ion leakage and ultimately cell death (Aoun 2000). Indeed, overexpression of *ERG26* and *ERG6* results in increased susceptibility to amphotericin B (Bhattacharya, Esquivel, and White 2018). Here, upon treatment with 0.25 µg/mL amphotericin B, the Δ*ire1* mutant was observed to have better growth compared to the Δ*ire1* + *IRE1* (Figure 3.7 B, C). This result further supports that the Δ*ire1* mutant has reduced ergosterol synthesis as the low expression of ergosterol would decrease the binding of amphotericin B to the cell membrane thereby conferring greater resistance to amphotericin B in the mutant strain. Moreover, it is possible that the downregulation of ergosterol results in the activation of secondary pathways which stabilizes the integrity of the membrane and makes the cell more resistant to amphotericin B.

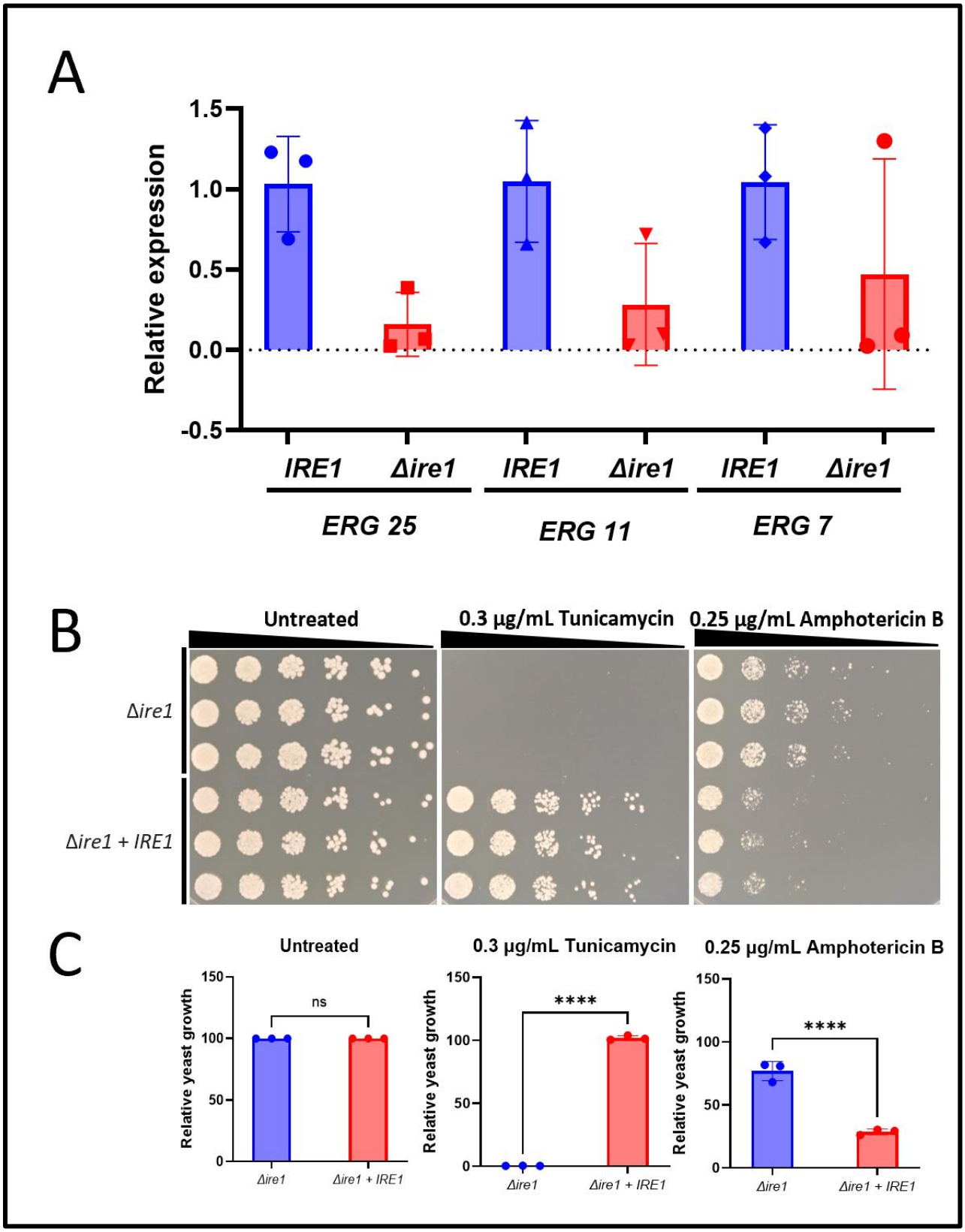

A
Relative expression
1.5
1.0
0.5
0.0
-0.5
IRE1
Δire1
IRE1
Δire1
IRE1
Δire1
ERG 25
ERG 11
ERG 7
B
Untreated
0.3 µg/mL Tunicamycin
0.25 µg/mL Amphotericin B
Δire1
Δire1 + IRE1
C
Untreated
0.3 µg/mL Tunicamycin
0.25 µg/mL Amphotericin B
Relative yeast growth
150
100
50
0
ns
****
****
Δire1
Δire1 + IRE1

Figure 3.7: Ergosterol synthesis gene expression is downregulated in the absence of Ire1.

(A) RNA of the early log phase Δ*ire1 + IRE1* culture was extracted, and qRT-PCR was performed to measure levels of *ERG25*, *ERG11* and *ERG7*. The relative expression levels were analyzed using the comparative ΔΔCt method with *ACT1* as a gene for normalization and graphed using GraphPad (Prism).

(B) For phenotypic comparison, fivefold serial dilutions of 0.1 OD_{600} cell suspensions of Δ*ire1* and Δ*ire1 + IRE1* were spotted onto YPD plates supplemented with 0.3 µg/mL tunicamycin, and 0.25 µg/mL amphotericin B. Growth of strains on the untreated plate were used as controls. Plates were incubated at 30 ˚C for 2 days and imaged using a mounted camera.

(C) Quantification of growth plates shown in B. Gray values of the 5× dilution of each strain in all plates were measured with ImageJ. Relative growth was calculated and graphed in a scatter dot plot with line at mean with SD, n = 3. ***$P < 0.0001$, ns $P > 0.9999$

Chapter 4

4 Discussion, and Conclusion

4.1 Discussion

In immunocompromised individuals, fungal infections are a common cause of mortality, and antifungal drug resistance is a growing threat to effective treatment (reviewed in (Fisher et al. 2022)). Consequently, the development of adequate antifungal treatments is imperative to combat one of our generation's most important health concerns. However, drug development first requires the understanding of these fungal pathogens and how they survive when subjected to existing antifungal compounds.

In my study, while I have found that the presence of the Ire1 protein is required for azole resistance, the canonical Hac1-dependent UPR transcriptional response was dispensable. Therefore, for the first time in *S. cerevisiae*, I have discovered a function of Ire1 that is independent of the Hac1 and its UPR-transcriptional response which confers greater tolerance to azole stress. To analyze this phenotype further, I employed Ire1 mutants which mechanistically abolish various functions of the protein. Through this, I found that both the kinase domain (required for UPR attenuation) and luminal domain (required for proteotoxic stress detection) are dispensable for azole resistance. Moreover, through the use of Kar2-GFP and a Δ*hrd1* mutant, I found that UPR regulation and the Hrd1 dependent ERAD pathway are not required for azole resistance. Indirectly, these results suggests that azoles likely do not lead to proteotoxic stress as Kar2 and Hrd1 dependent ERAD are vital for proteotoxic stress mediation. Moreover, as Ire1 activates a distinct UPR-transcriptional response upon LBS detection (Ho et al. 2020b), and azole treatment did not result in the activation of the UPR, we can also infer that azoles do not result in LBS that is detected by Ire1. To validate this, future experimentation with Ire1 transmembrane domain mutants such as R537Q and V535R (Ho et al. 2020b; Duc, Takagi, and Kimata 2018) which inhibit/reduce the detection of LBS should be pursued.

Interestingly, my results suggest that the absence of Ire1 within the ER membrane leads to the downregulation of ergosterol synthesis genes. This downregulation of ergosterol synthesis genes in Ire1 deficient cells has also been observed in both *C. albicans* (Sircaik et al. 2021) and *Aspergillus fumigatus* (X. Feng et al. 2011). However, it is yet to be determined if this transcriptional downregulation directly results in the reduced expression of ergosterol in yeast membranes. The late pathway enzymes encoded by *ERG2* to *ERG6* have low substrate specificity and accept a broad range of similar structures (Joshua and Höfken 2017), therefore, it is possible that the downregulation of certain *ERG* genes may allow the accumulation of a mixture of sterols instead of only a certain enzyme's substrate and not necessarily lead to the downregulation of ergosterol in the membranes. Currently, the relationship between Ire1 absence and ergosterol downregulation requires further experimental exploration. Therefore, direct analysis of ergosterol levels in the cell and ER membranes of Δ*ire1* mutant is required, possibly through the use of dehydroergosterol (DHE) which is a fluorescent sterol closely related to ergosterol and has been used to measure the relative abundance of sterols within membranes of yeast cells (Solanko et al. 2018). Furthermore, to analyze the different sterols within the global lipidomes of yeast, gas chromatography-mass spectrometry (GCMS) based tools for the determination of sterol structure and quantification have also been suggested (Singh et al. 2020).

Due to the localization of both Ire1 and late pathway Erg enzymes in the ER membrane, it is possible that the loss of the ER resident Ire1 which likely induces membrane stress, results in the downregulation of other ER resident proteins. Interestingly, transcriptome analysis by Ho et al., revealed that ergosterol synthesis genes are not regulated by the UPR (Ho et al. 2020b). Therefore, compromising UPR by *IRE1* deletion leads to ergosterol downregulation via activation and/or repression of other signaling pathways. It may be suggested that the absence of Ire1 which

leads to the loss of basal levels of *HAC1* splicing generates a change in the regulation of a secondary pathway important for ergosterol biosynthesis. However, the Δ*hac1* mutant which also lacks UPR does not show an increase in sensitivity to azoles, indicating that basal levels of *HAC1* splicing are not required. To ensure that *HAC1* deletion leads to *ERG* downregulation, further experimentation is required. Alternatively, it is possible that the absence of Ire1 leads to the regulation of other stress pathways controlling ergosterol synthesis enzymes. For example, ergosterol synthesis is transcriptionally controlled by the HOG stress pathway which is responsible for the adaptation of hyperosmotic stress (Montañés, Pascual-Ahuir, and Proft 2011). However, in the absence of Ire1, the HOG pathway could not be involved in the downregulation of ergosterol as Ire1 and Hac1 are both required for Hog1 kinase-dependent transcriptional regulation upon ER stress (Bicknell, Tourtellotte, and Niwa 2010) (Figure 4.1). Currently, apart from the HOG pathway, there is a gap in knowledge concerning mechanisms involved in both ergosterol biosynthesis and ER stress adaptation. Therefore, study of both the wild type and Δ*ire1* mutant transcriptome, possibly through RNA-sequencing is warranted to identify alterations in stress pathways in the absence of Ire1. Alternatively, a genome-wide screen for suppressors of the Ire1 azole sensitivity could help elucidate the mechanisms associated with the phenotype.

Interestingly, studies have found that the mammalian IRE1 binds to proteins other than BiP, such as the 80S ribosome with high affinity (Acosta-Alvear et al. 2018). Moreover, as Ire1 is a protein with a kinase domain which phosphorylates its ribonuclease domain, it is possible that other kinase and phosphatase proteins found within the cytosol interact with Ire1. However, other than the misfolded proteins within the ER lumen and Kar2, to my knowledge there are no documented interactions of proteins directly with Ire1 in yeast. Further exploration of this is warranted as unknown cytosolic protein interactions such as kinases or phosphatases may be

responsible for the phenotype observed with azole treatment. For example, it is possible that a cytosolic phosphatase prevents Ire1 from splicing the *HAC1* mRNA during azole treatment despite the membrane stress induced by azoles. To investigate this, pull-down assays which determine the physical interaction between two or more proteins could be pursued.

An anomaly of the growth assays in this study is that colonies within a single dilution factor are observed to have a variety of differences in size, ex. figure 3.1 A, 3.4A, 3.5 A, 3.6 B, 3.7B. Variability between cells within a strain derived from a single colony is possible due to a wide range of possibilities such as slight mutations within the genome and epigenetic effects due to chromatin accessibility. Indeed, studies have implicated epigenetic mechanisms in drug resistance observed in fungi (reviewed in (Chang et al. 2019)) and may explain the slight differences in colony size observed in the growth assays here. Slight variance between individual cells of a strain are expected, and has been controlled for through experimentation with multiple biological replicates.

Since essential processes related to growth, virulence and UPR of pathogenic species are not necessarily shared with the budding yeast (Skrzypek et al. 2017; Demuyser and Van Dijck 2019; Hernández-Elvira et al. 2018), direct extrapolation of data from this study to other species becomes difficult. Therefore, I will next discuss some key features of the non-pathogenic *Schizosaccharomyces pombe* and the pathogenic *Candida albicans*, *Candida glabrata*, and *Cryptococcus neoformans*, and how the existing data of these species compare with my findings here (summarized in table 4.1).

Table 4.1: Summary of similarities and differences between species discussed.

Species name	Pathogenic	*HAC1* orthologue	UPR	RIDD	Transcriptional program/s activated upon ER stress	Response to azole antifungals
Saccharomyces cerevisiae	No	Yes	Yes	No	UPR	No UPR activation. Upregulate sterol influx and drug efflux (MDR) proteins
Schizosaccharomyces pombe	No	No	No	Yes	Stress-activated kinase pathway	No UPR activation. MDR (upregulation of drug efflux proteins)
Candida albicans	Yes	Yes	Yes	?	UPR, Calcineurin pathway	No UPR activation. MDR
Candida glabrata	Yes	Yes, but not spliced by Ire1	No	Yes	Calcineurin, and Slt2 MAPK pathway	No UPR activation. MDR
Cryptococcus neoformans	Yes	Yes, known as *HXL1*	Yes	No	UPR	UPR, Disomy of chromosome 1 and others

Note: Pathways listed in columns 6 and 7 are not exhaustive, and likely involves more than what is indicated for some species.

4.1.1 *Schizosaccharomyces pombe*

Although *S. cerevisiae* and *S. pombe* share the designation "yeast," and are both models for biomedical research, these two species are evolutionarily quite distinct due to their divergence from a common ancestor estimated at one billion years (Heckman et al. 2001; Hedges 2002). Moreover, unlike the budding yeast *S. cerevisiae*, *S. pombe* is a fission yeast and is an important model organism in the study of cell division and sexual differentiation (Forsburg 2005). Therefore, work with both species offers researchers complementary approaches and insights.

The Ire1 of *S. pombe* (*Sp*Ire1) possesses the similar structural features of *S. cerevisiae* (Hernández-Elvira et al. 2018), yet, the residues important in the RNA docking site of the ribonuclease domain that are well conserved in other yeast species are not present in the *Sp*Ire1 (Mori et al. 1993; Shamu and Walter 1996; van Anken et al. 2014). Furthermore, there is no specific substrate for Ire1 identified in *S. pombe*, indicating that this species lacks a *HAC1* orthologue (Hernández-Elvira et al. 2018). Despite this, *Sp*Ire1 null mutants are sensitive to ER stress, and instead activate regulated Ire1 dependent degradation (RIDD) upon the induction of ER stress (Kimmig et al. 2012). Curiously, approximately 31 % of the mRNAs degraded through RIDD by *Sp*Ire1 code for lipid metabolism genes, specifically, sterol metabolism. This indicates that in *S. pombe*, ER stress is mediated by the downregulation of sterol biosynthesis. This is similar to the observations of my study, where in the absence of *S. cerevisiae* Ire1 (*Sc*Ire1), which likely induces ER stress, ergosterol biosynthesis is downregulated. In *S. pombe*, although it is unknown how the reduction in sterol synthesis counteracts ER stress toxicity, downregulation of sterol biosynthesis has been suggested to stabilize ER membrane fluidity (Kimmig et al. 2012). Specifically, in mammalian cells, ER stress limits sterol exit through vesicular transport and leads to higher rigidity of the membrane due to cholesterol accumulation (Nilsson et al. 2001; B. Feng

et al. 2003). Therefore, in *S. pombe*, to maintain appropriate membrane fluidity which sustains basic ER functions, compensation is suggested by Kimmig and co-workers to occur through RIDD by the degradation of mRNAs which code for sterol synthesis genes (Kimmig et al. 2012).

I posit that somewhat of a similar process may occur in *S. cerevisiae*, where the absence of Ire1 induces ER stress in the form of membrane sterol accumulation which requires ergosterol downregulation to maintain appropriate membrane fluidity (Figure 4.1). However, this downregulation becomes detrimental to the Δ*ire1* cells upon treatment with azoles which further disrupt ergosterol biosynthesis. To explore this further, as mentioned before, the levels of sterols directly in the *S. cerevisiae* ER membranes of both Δ*ire1* and wild type *IRE1* should be analyzed. *S. pombe* posses a robust multidrug resistance (MDR) transcriptional response which upregulates drug efflux proteins (Arita et al. 2011; Wolfger, Mamnun, and Kuchler 2001). Deletion of genes which code for major facilitator superfamily pumps and ATP-binding cassette transporters, resulting in the suppression of the multi drug resistance (MDR) system, increases sensitivity to azoles (Kawashima et al. 2012). In *S. cerevisiae*, the MDR drug efflux system is also known to be an important pathway used to confer drug resistance (De Hertogh et al. 2002). In mammalian cells, it is known that the UPR regulates the expression of MDR efflux proteins (Gao et al. 2020), however, such regulation in *S. cerevisiae* is unclear. Therefore, future experimentation should pursue the levels of MDR pumps in the absence of Ire1 in *S. cerevisiae* to determine the link between Ire1 downregulation and azole sensitivity.

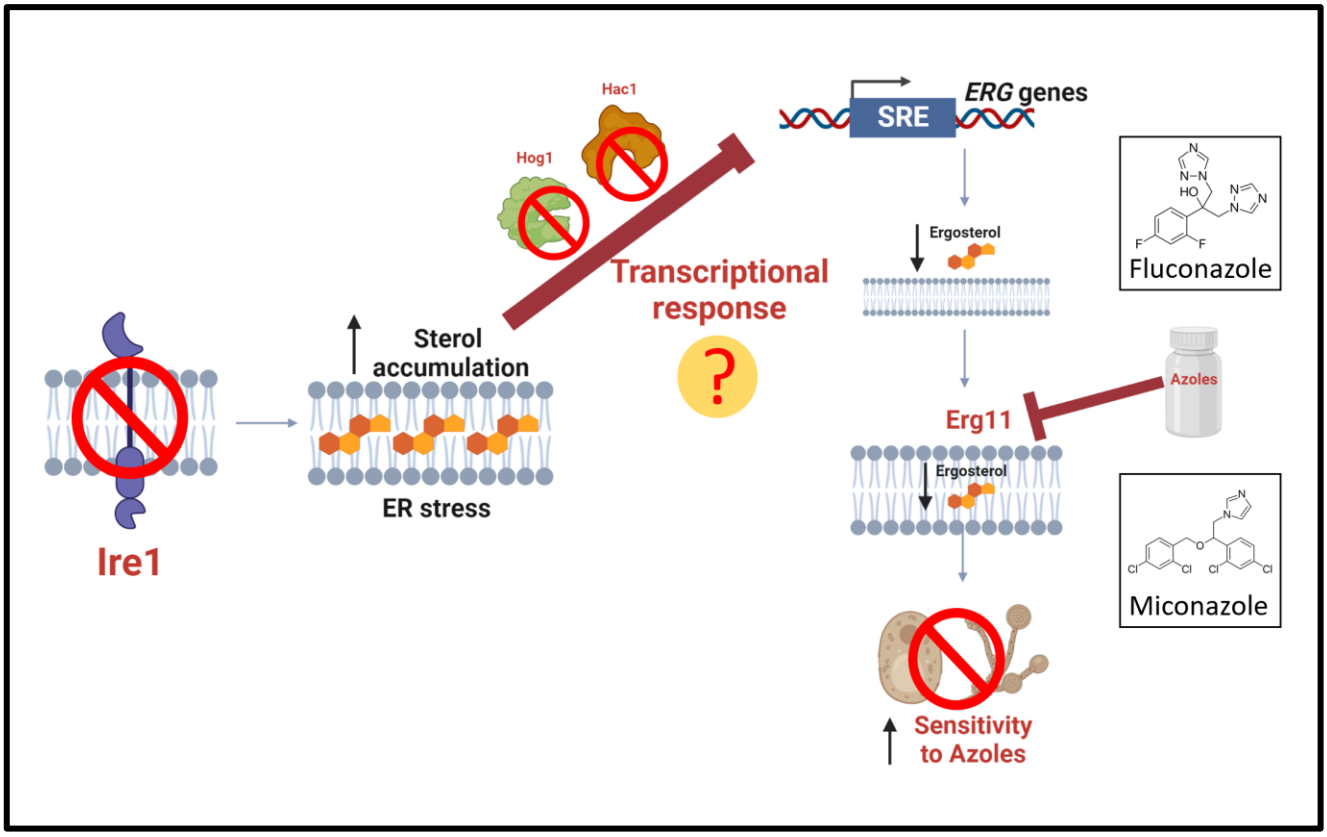

Hac1
Hog1
SRE
ERG genes
Transcriptional response
?
Ergosterol
Fluconazole
Sterol accumulation
Azoles
Erg11
Ergosterol
Ire1
ER stress
Miconazole
Sensitivity to Azoles

Figure 4.1: Schematic of proposed model on the link between Ire1 deletion and increased sensitivity to azoles.

I propose that the absence of Ire1 within the ER membrane causes ER stress in the form of sterol accumulation which leads to the downregulation of ergosterol as a method of compensation through an unknown transcription program. The UPR system mediated by the Hac1 transcription factor cannot be the cause for ERG gene downregulation as Hac1 does not bind to the SRE. Moreover, the HOG transcriptional response mediated by the Hog1 kinase is also not likely the cause of ERG gene downregulation as both Ire1 and Hac1 are required for the phosphorylation of Hog1 upon induction of ER stress. Nevertheless, this compensation becomes detrimental to the Ire1 deficient cells upon treatment with azoles which further disrupt ergosterol biosynthesis. (Image created with BioRender)

4.1.2 *Candida albicans*

Like many other species of the *Candida* genus, *C. albicans* is part of the human commensal microbiota and found in a broad range of tissues including the gastrointestinal tract (Schulze and Sonnenborn 2009). Yet in the event of homeostatic imbalance, *C. albicans* becomes an opportunistic fungal pathogen that can develop into a systemic infection and cause for mortality (Zaoutis et al. 2005). *C. albicans* in particular accounts for over 40 % of all fungal infections and therefore is the most studied species of this genus (Angoulvant, Guitard, and Hennequin 2016; Gabaldón and Fairhead 2019). Furthermore, the Centres for Disease Control (CDC) has recognized the emerging prevalence of fluconazole resistance displayed by *Candida* species as a serious threat to human health ("Antifungal Resistance | Fungal Diseases | CDC" 2022).

The evolutionary divergence of *C. albicans* from *S. cerevisiae* is estimated at 300 million years (Hedges et al. 2015), yet *C. albicans* contains a typical Ire1 protein with similar structural domains as *Sc*Ire1, including conserved residues within the kinase and RNase domains (Hernández-Elvira et al. 2018). Of significance, the *C. albicans* Ire1 (*Ca*Ire1) has been shown to be vital for azole resistance in *C. albicans* (Sircaik et al. 2021). However, in contrast to my findings with *Sc*Ire1 kinase domain, unpublished data from our lab suggests that the kinase domain of *Ca*Ire1is required for azole tolerance. This indicates that unlike in *S. cerevisiae*, azole treatment may result in either proteotoxic or LBS thereby activating the *Ca*Ire1 for the counteraction of azole stress. However, a recent study has found that although *Ca*Ire1 is required for mediation of stress caused by fluconazole, *C. albicans HAC1* (*CaHAC1)* splicing was not detected, indicating no activation of the UPR transcriptional response (Sircaik et al. 2021). This is similar to my findings with *S. cerevisiae* as *C. albicans* also does not utilize its functional UPR system for azole resistance.

Azole treatment in *C. albicans* is thought to activate RIDD, however this is not validated and is an area that remains to be explored. Since the Ire1 kinase domain is required for azole resistance (Tam, Koong, and Niwa 2014), there is a possibility that RIDD is activated. Furthermore, as the downregulated *IRE1* (*ire1*DX) mutant of *C. albicans* was observed to decrease expression of ergosterol synthesis genes (Sircaik et al. 2021), it is possible that RIDD selectively degrades mRNA for ergosterol synthesis enzymes similar to *S. pombe*. Alternatively, it is also possible that *Ca*Ire1 is required for azole resistance in a UPR and RIDD independent manner, similar to that of *Sc*Ire1 (depicted in Figure 4.1). Therefore, to determine the mechanism utilized by *C. albicans* for azole resistance, future experimentation that pursues the level of ergosterol in both the ER membrane and plasma membrane of *C. albicans ire1*DX mutant is warranted to draw parallels with *S. cerevisiae.*

In addition to the possibility of RIDD activation upon azole treatment, *Candida* species are also known to activate the upregulation of drug efflux proteins (Mane et al. 2016; Maebashi et al. 2001; Sanglard et al. 1999), similar to *S. pombe* and *S. cerevisiae*. Therefore, measuring levels and activity of these efflux proteins in the absence and presence of Ire1 during azole treatment in *S. cerevisiae* would provide valuable information on the mechanism utilized for azole resistance.

4.1.3 *Candida glabrata*

C. glabrata is to some extent commensal, yet is an established fungal pathogen in humans (Gabaldón and Fairhead 2019). In recent years, the increased incidence of *C. glabrata* infections has been attributed to this pathogen's inherent higher resistance to commonly used antifungals (Angoulvant, Guitard, and Hennequin 2016). Despite its genus name, *C. glabrata* is more closely related to *S. cerevisiae* than other *Candida* species as its evolutionary divergence from *S. cerevisiae* is estimated at 100-300 million years (Dujon et al. 2004; Wolfe and Shields 1997). In

contrast, the evolutionary divergence of *C. glabrata* from *C. albicans* is estimated to be at 700-800 million years (Hedges et al. 2004).

C. glabrata Ire1 contains the typical Ire1 domains as in *S. cerevisiae*, and possesses an orthologue of *HAC1* (Hernández-Elvira et al. 2018). However, *C. glabrata* Ire1 (*Cg*Ire1) does not seem to splice *C. glabrata HAC1* (*CgHAC1*) upon treatment with proteotoxic stress inducers, and instead solely relies on the RIDD pathway, similar to *S. pombe* (Miyazaki et al. 2013). Therefore, *CgHAC1* remains unspliced in both stressed and non-stressed conditions. Furthermore, unlike *CgIRE1* null mutants, *CgHAC1* null mutants do not show more sensitivity to ER stress inducers, indicating the dispensable nature of *CgHAC1* during ER stress. However, as current literature has not validated the activation of RIDD upon treatment with azoles, it is possible that *Cg*Ire1 confers drug resistance similar to *Sc*Ire1. To validate this, future experimentation in *C. glabrata* with a ribonuclease mutant of *Cg*Ire1 which abolishes RIDD activity should be pursued.

4.1.4 *Cryptococcus neoformans*

The divergence between the Ascomycota phyla (includes *S. cerevisiae*) and the Basidiomycota phyla (includes *C. neoformans*) is estimated at 500 million years (Taylor, Hass, and Kerp 1999; Stajich et al. 2009). Yet, studies have demonstrated the expression of a large fraction of conserved *S. cerevisiae* genes periodically in *C. neoformans* as both species are budding yeast (Kelliher et al. 2016). Despite the genetic similarity, *C. neoformans* is pathogenic and is the most common cause of severe pulmonary infections and meningoencephalitis in immunocompromised patients (Hernández-Elvira et al. 2018; Denham and Brown 2018).

In comparison to *Sc*Ire1, *C. neoformans* Ire1 (*Cn*Ire1) is structurally conserved along with the important kinase and the RNase domain residues (Hernández-Elvira et al. 2018). However, in comparison to *S. cerevisiae* and other species discussed here, *C. neoformans* possesses the lowest

conserved DNA binding domain in the Hac1 orthologue (known as Hxl1). Moreover, unlike in some other species, the unspliced version of the *HXL1* can be translated into a protein (Cheon et al. 2014). Yet, *Cn*Ire1 dependent *HXL1* splicing is essential for resistance to proteotoxic ER stress inducers like DTT and Tunicamycin (Cheon et al. 2011). Therefore, *HXL1* splicing during ER stress is predicted to be regulated through the interaction of an mRNA binding protein known as Puf4 (Glazier et al. 2015). Apart form the ER stress response, *Cn*Ire1 has a variety of other functions which includes biosynthetic regulation of the antiphagocytic capsule and antifungal drug resistance, therefore, UPR signaling is critical for virulence in *C. neoformans.*

Unfortunately, due to the widespread use of antifungals, a recent study found that over 30 % of the *C. neoformans* isolates were no longer susceptible to fluconazole and other common antifungals (Smith et al. 2015). Therefore, research pertaining to antifungal drug resistance of this species is of utmost importance. Research shows that Δ*ire1* and Δ*hxl1* mutants of *C. neoformans* show significantly enhanced susceptibility to various azoles including fluconazole, indicating that the Hxl1-dependent UPR pathway is essential (Cheon et al. 2011). Furthermore, a study found that azole treatment is sensed as ER stress and subsequently activates the Ire1-dependent *HXL1* splicing and *KAR2* induction by the UPR pathway (Jung, Kang, and Bahn 2013). Interestingly, unlike my discovery here with *S. cerevisiae* and all the other species discussed here, *C. neoformans* is the only species that has been observed to splice its *HAC1* orthologue and activate the UPR transcriptional response upon treatment with azoles.

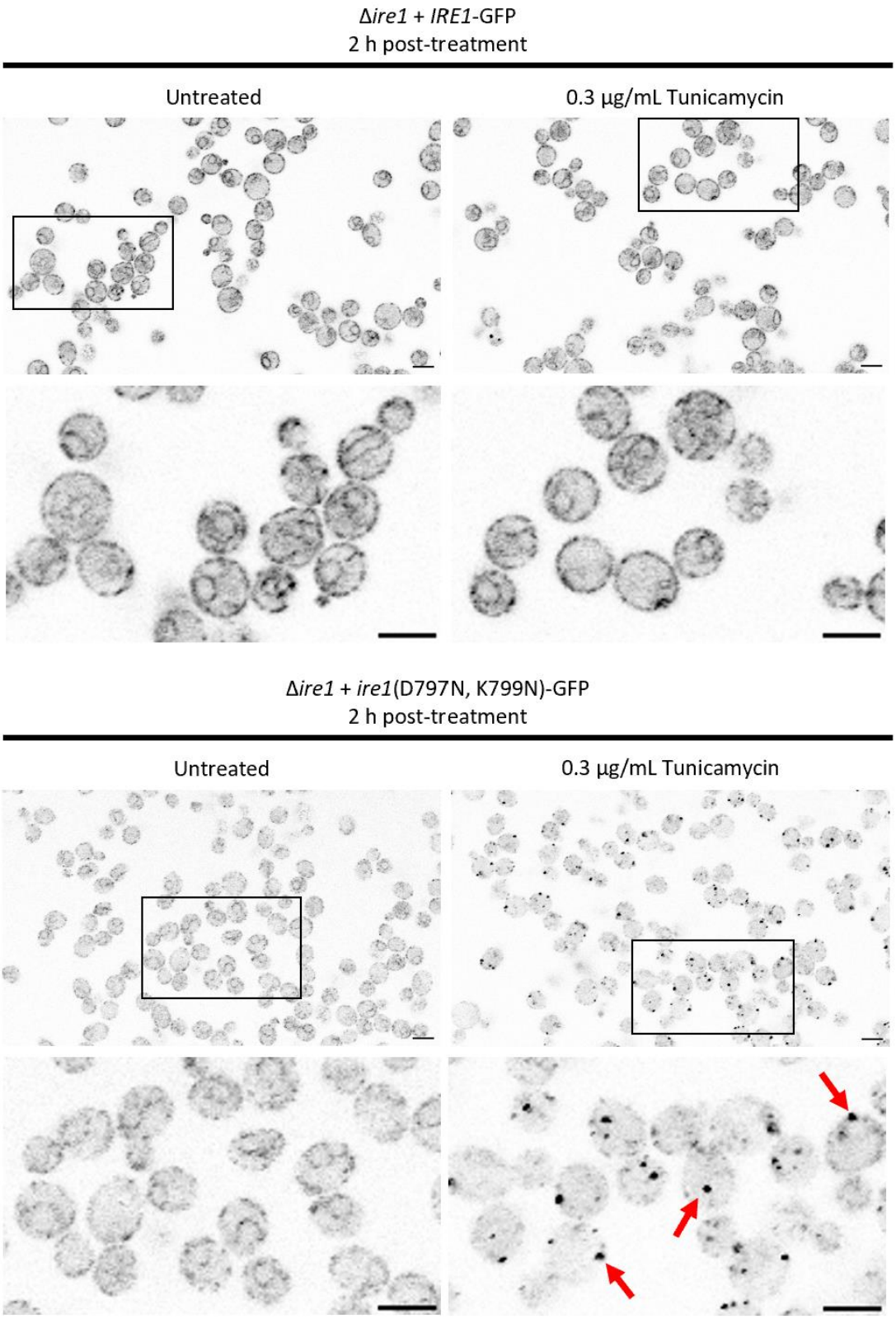
4.2 Conclusion

Unlike in *S. cerevisiae*, the Ire1 of pathogenic species like *C. albicans* and *C. glabrata* possess a function independent to the UPR-transcriptional response known as RIDD. Additionally, certain aspects of azole susceptibility and genes involved in azole resistance are not shared between the pathogens and *S. cerevisiae* (Mount et al. 2018). This makes drawing parallels between *S. cerevisiae* and pathogenic species quite difficult. However, comparison of the existing data on pathogenic species has shown some similarities to *S. cerevisiae*. For example, some pathogenic species do not utilize the UPR transcriptional program for azole resistance. Moreover, it is possible that *C. albicans* utilizes Ire1 in a manner similar to *S. cerevisiae*, as RIDD is not confirmed, and *CaHAC1* splicing has not been observed despite the requirement of *Ca*Ire1 upon treatment with azoles. Additionally, this species has demonstrated the downregulation of ergosterol synthesis genes in *Ca*Ire1 deficient cells. Therefore, it is possible that the absence/ deficiency of Ire1 within the ER membrane causes ER stress in the form of sterol accumulation which leads to the downregulation of ergosterol as a method of compensation (Figure 3.6). However, to validate this hypothesis, future experimentation which determines the level of sterols in Ire1 deficient cells in both *S. cerevisiae* and *C. albicans* is required.

In conclusion, this study provides mechanistic insight into the function of Ire1 in *S. cerevisiae*, and further the understanding of the difference between *S. cerevisiae* and other yeast species. Interestingly, several studies link the presence of pathogenic yeast species with gut diseases such as Chron's disease, diarrhea and pouchitis, and the presence of *S. cerevisiae* with a healthy gut (Kühbacher et al. 2006; Hoarau et al. 2016; Sangster et al. 2016; Sokol et al. 2017). Therefore, there may be an interest to develop new antifungal drugs which act only against the pathogenic species without affecting levels of harmless commensal *S. cerevisiae* populations of

the gut (Demuyser and Van Dijck 2019). As such, my study which provides mechanistic insight into the function of Ire1 in *S. cerevisiae* during azole resistance would certainly be useful.

4.3 Supplementary figure



Supplementary figure 1. The kinase mutant induces Ire1 clustering when treated with 0.3 µg/ml tunicamycin, unlike the *IRE1* complemented strain.

Early log phase aliquots of Δ*ire1* + *IRE1* and Δ*ire1* + *ire1*(D797N, K799N) cells were treated with 0.3 µg/mL tunicamycin for 2 h and imaged using confocal microscopy. Red arrows point to the Ire1(D797N, K799N)-GFP clusters. Scale bars represent a length of 5 µm.

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