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Regulation of Endoplasmic Reticulum Stress in Saccharomyces cerevisiae

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Abstract

The budding yeast *Saccharomyces cerevisiae* has been used extensively to uncover the genetic mechanisms that control basic cellular processes, including survival, maintenance, and response to stressors. One metric of yeast survival is chronological lifespan (CLS), which is the amount of time non-dividing yeast cells can survive at stationary phase. Variations in CLS following genetic alteration are used to understand the function of specific genes and pathways in cellular aging. Many factors contribute to aging, including accumulation of toxic misfolded secretory proteins in the endoplasmic reticulum (ER stress), to which the cell responds through activation of ER stress signaling pathways, such as the Unfolded Protein Response (UPR).

In this thesis, we first developed new fluorescence-based assays and a corresponding software program to measure yeast CLS and investigate how ER stress responses impact yeast CLS. Using these assays, we found that inositol was important for CLS, especially in cells with compromised UPR. We also found that UPR-dependent upregulation of the ER chaperone Kar2 is critical for CLS, and we demonstrate that deletion of ER-associated degradation (ERAD) components accelerates chronological aging. We argue that the capacity of the ER quality control machinery to degrade misfolded secretory proteins is an important determinant of ER stress sensitivity and, by extension, yeast CLS. Finally, we aimed to identify ways to modulate ER stress and the UPR; we worked to decipher the mechanism of a compound previously hypothesized to act as a "chemical chaperone" to alleviate ER stress by directly improving protein folding, trafficking, and degradation – tauroursodeoxycholic acid, or TUDCA. We found that, while TUDCA alleviates ER stress, this process can be uncoupled from UPR signaling. We also found little evidence that TUDCA works as a true chemical chaperone. By contrast, it alleviates ER stress indirectly through activation of another stress response, the Cell Wall Integrity (CWI) pathway. We thus discovered a novel mechanism of modulating ER stress. Overall, this thesis has identified new factors influencing ER stress regulation, including chronological aging, protein quality control mechanisms, the CWI pathway, and small molecules such as TUDCA.

Keywords

chronological lifespan, aging, yeast, fluorescence, unfolded protein response, caloric restriction, endoplasmic reticulum stress, ER-associated degradation, cell wall integrity, TUDCA, chemical chaperone, protein quality control, proteostasis

Summary for Lay Audience

In order to survive, all living things must be able to respond to insults, or stressors, in their environments. Some common stressors include starvation, toxins, and aging. During aging, cells can also become more sensitive to all other stressors. This can result in age-related diseases, such as Huntington's disease, Alzheimer's disease, and type 2 diabetes. In this thesis, the first goal was to determine why this occurs. Using a budding yeast model, we first developed new methods to study yeast aging with existing equipment and a new software program. From there, we were able to identify cellular processes and genes that determine a cell's lifespan. These included the cell's natural stress responses (such as the Unfolded Protein Response, or UPR), nutrient levels and pH, and its built-in quality control processes (such as the breakdown of damaged or misfolded proteins). The loss or alteration of any of these, especially when combined with a specific form of stress known as Endoplasmic Reticulum Stress (ER stress), resulted in a short lifespan. Finally, using this information, we aimed to find ways to change cellular sensitivity to ER stress, which could be used to treat age-related diseases. One way to do so utilized a traditional Chinese medicine called TUDCA (a chemical originally derived from bear bile) which is currently in clinical trials for several diseases worsened by aging and ER stress. Despite success in treating these diseases, TUDCA's mechanism is still a mystery. We hypothesized that it may work by altering the cell's stress responses to make the cell less sensitive to additional stress. We found that it reduced stress response activation via the UPR, but that it could still reduce stress sensitivity in cells lacking the UPR. It seemed to work from another, unexpected angle: a quality control mechanism that usually maintains yeast cell wall integrity (CWI). This indirect way of altering stress sensitivity could offer a new target for developing stress-reducing drugs. Overall, this thesis discovered new information about what occurs during aging and cellular stress exposure and identifies new ways in which these processes can be chemically altered.

Co-Authorship Statement

Chapter 2: Parnian Etedali-Zadeh and Anna E. Park provided the cell counter images in Figure 2.2B. Sonja DiGregorio provided the spot assay in Figure 2.10A. I performed all other experiments. The ANALYSR software was developed by myself and A. Demetri Pananos, who also adapted it for use in a Python script editor. Martin L. Duennwald assisted in the conception of some of the experiments and provided critical revision. Patrick Lajoie was the supervising author and also co-wrote some sections of the manuscript.

Chapter 3: Julie Genereaux assisted in the generation of some deletion strains. Martin L. Duennwald provided the UPRE-LacZ and CFU data in Figure 3.4, Julie Genereux provided the spot assays in Figure 3.5A, and Parnian Etedali-Zadeh provided the survival curve in Figure 3.5D. I performed all other experiments. Parnian Etedali-Zadeh, Elena N. Fazio, Julie Genereux, and I generated the data and analyzed the results with Patrick Lajoie. Patrick Lajoie, Martin L. Duennwald and I wrote the manuscript.

Chapter 4: Julie Genereaux assisted in the generation of some deletion strains. Patrick Lajoie provided the fluorescent microscopy images in Figure 4.1C and S4.5A, *HAC1* splicing assay in Figure 4.3B and C, the deletion library screen in Figure 4.6A and B, and the growth assays in Figure 4.7, S4.1A and B, and S4.2. I performed all other experiments. Robyn D. Moir, Ian M. Willis, and Erik L. Snapp contributed to the conception of some of the experiments. I wrote the manuscript, with assistance and edits from Patrick Lajoie and Martin L. Duennwald.

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List of Abbreviations

µg/mL	Micrograms per millilitre
µM	Micromolar
4-PBA	4-Phenylbutyric acid
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
ANALYSR	Analytical Algorithm for Yeast Survival Rates
Ca^{2+}	Calcium
Casp	Caspofungin
CDRE	Calcineurin-dependent response element
CFU	Colony-forming units
CFW	Calcofluor white
CLS	Chronological lifespan
CR	Caloric restriction
CWI	Cell wall integrity
D_2O	Deuterated water
DAmP	Decreased abundance by mRNA perturbation
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
ERSU	ER stress surveillance pathway
GFP	Green fluorescent protein
h	Hour
HD	Huntington's disease
HSR	Heat Shock Response
Htt	Huntingtin protein
mM	Millimolar
mRNA	Messenger ribonucleic acid

O/N	Overnight
OD	Optical density
PBS	Phosphate buffered saline
PI	Propidium iodide
PolyQ	Polyglutamine
PQC	Protein quality control
RFP	Red fluorescent protein
RIDD	Regulated Ire1-dependent decay
RLS	Replicative lifespan
rRNA	Ribosomal ribonucleic acid
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SC	Synthetic complete (media)
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
STRE	Stress responsive element
Tm	Tunicamycin
TMAO	Trimethylamine N-oxide dihydrate
TUDCA	Tauroursodeoxycholic acid
UDCA	Ursodeoxycholic acid
UPR	Unfolded Protein Response
UPRE	Unfolded Protein Response Element
WT	Wild-type
YPD	Yeast-Peptone-Dextrose (media)

Chapter 1

1 Introduction

Sections of this chapter have been extracted from Chadwick and Lajoie (2019).

1.1 Protein Homeostasis and Response to Cellular Stress

Protein homeostasis (or proteostasis) is the sum of cellular processes involved in the "life cycle" of a protein: DNA transcription, mRNA translation, and subsequent protein folding and eventual degradation (Balch et al., 2008). In order for a cell to remain functional and able to adapt to changing biochemical and environmental signals, proteostasis must remain uncompromised (Ben-Zvi et al., 2009). Protein folding is particularly important for cellular processes, as the final conformation of a folded protein is essential to its function.

Under normal circumstances, proteins destined for the secretory pathway are translated directly into the endoplasmic reticulum, bound by chaperone proteins, folded, and then packaged into vesicles for secretion (Novick et al., 1981). In some cases, however, this pathway can go awry; proteins may become misfolded or unfolded, and unable to be recovered by the protein quality control machinery. In this instance, the improperly folded protein is targeted for degradation, exported into the cytosol, and degraded by a proteasome (Werner et al., 1996). Again, however, this process is imperfect. Some environmental, cellular, or molecular factors can cause disruptions in this pathway, preventing the proper turnover of misfolded or unfolded proteins, potentially leading to their accumulation and aggregation. This generates a cellular condition known as ER stress (Friedlander et al., 2000).

ER stress and the failure to correctly fold proteins are associated with loss of protein function and cell death (Hetz et al., 2006; Upton et al., 2012; Zinszner et al., 1998). To avoid this, the cell resolves misfolded protein stress via two major stress response pathways: the heat shock response (HSR) (Verghese et al., 2012), which handles misfolded proteins in the cytoplasm, and the unfolded protein response (UPR), which

takes place in the endoplasmic reticulum (ER) (Cox and Walter, 1996; Kohno et al., 1993; Liu and Chang, 2008). These protein quality control mechanisms are essential for maintaining the function and integrity of cellular processes. When perturbed, they can lead to whole-cell dysfunction and toxicity (Ruis and Schüller, 1995; Voellmy, 2004). Under normal conditions, both lead to resolution of the cellular stress caused by the presence of misfolded proteins. In some cases, such as in several misfolded protein-associated diseases (Torres et al., 2015; Yoshida, 2007), these stress response pathways themselves can become impaired. This leads to further accumulation of misfolded proteins, which in turn causes further UPR or HSR impairment (Delépine et al., 2000; Zhang et al., 2002). Misfolded protein aggregates have also been shown to bind machinery important for degrading misfolded proteins via ER-associated degradation (ERAD), a protein quality-control mechanism, which recognizes unfolded or misfolded proteins synthesized in the endoplasmic reticulum (Lippincott-Schwartz et al., 1988; McCracken and Brodsky, 1996). This ERAD impairment induces further stress in the ER and causes induction of the UPR. Proteostatic dysfunction essentially leads to a vicious cycle of increasing ER stress, protein accumulation, and stress response impairment.

The UPR is of particular interest to this research because of its well-documented, but poorly understood, involvement in several disease pathogeneses. The UPR is a complicated signaling pathway which works to resolve ER stress and allow protein synthesis and folding to continue, and has been shown to intersect with multiple cellular pathways and processes to do so, including (but not limited to) those occurring in the ER (Snapp, 2012; Travers et al., 2000; Welihinda et al., 1999). It has also been shown to be impacted by several seemingly unrelated external influences, including aging and lipid metabolism, and dysfunction in this pathway has been linked with shortened cellular lifespan and cell death (Hou et al., 2014; Jazwinski, 2002; Labunskyy et al., 2014). Because of this, the study of the molecular mechanisms behind ER stress and the UPR is essential to our understanding of how protein homeostasis impacts the entire cell and its processes, including response to stressors, aging, and cell death.

1.2 The Unfolded Protein Response

As previously mentioned, the UPR is a stress response pathway specifically activated in response to ER stress, which is a condition that can be generated by things such as small molecules, environmental factors, or the accumulation of misfolded proteins in the endoplasmic reticulum (Welihinda et al., 1999). The UPR is activated when ER stress sensors embedded in the ER membrane detect the stressors and respond. Interestingly, the ultimate function of the UPR depends on the degree of activation and the length of time that UPR signaling is maintained before the stress is resolved (Rutkowski and Kaufman, 2007; Rutkowski et al., 2006; Vidal and Hetz, 2012). It is primarily an adaptive response, which rescues cells from ER stress, but prolonged ER stress or a high degree of UPR signaling causes the response to become maladaptive. In these circumstances, the UPR can activate alternate signaling pathways that result in apoptosis (Bernales et al., 2006; Lin et al., 2007; Thibault and Ng, 2011).

In mammals, three distinct ER stress sensors exist: inositol requiring enzyme 1 (IRE1) (Calfon et al., 2002; Sidrauski and Walter, 1997; Yoshida et al., 2001), double-stranded RNA-activated protein kinase like endoplasmic reticulum kinase (PERK) (Harding et al., 2000a), and activating transcription factor 6 (ATF6) (Yoshida et al., 1998). Some major components of the UPR were first discovered in 1993, when IRE1 was identified as a connection between protein folding dysfunction in the ER lumen and the downstream effects seen in the nucleus (such as the transcription of UPR-associated genes) (Cox et al., 1993; Mori et al., 1993). At that point, however, it was not clear exactly how the two processes were linked. Hac1/XBP1 activation via splicing by IRE1 was eventually identified as the connecting mechanism, helping establish IRE1's specific role as an ER-bound stress sensor (Cox and Walter, 1996; Sidrauski and Walter, 1997). Since then, the roles and mechanisms for many of the UPR signaling pathway components have been identified and classified (**Figure 1.1**).

The three ER stress sensor pathways in mammalian cells are intertwined, but perform some distinct functions. In the absence of ER stress, all three are bound by the chaperone protein BiP/GRP78 (Kar2 in budding yeast) on their luminal domain, which represses their activity (Bertolotti et al., 2000; Kimata et al., 2004; Pincus et al., 2010; Shen et al.,

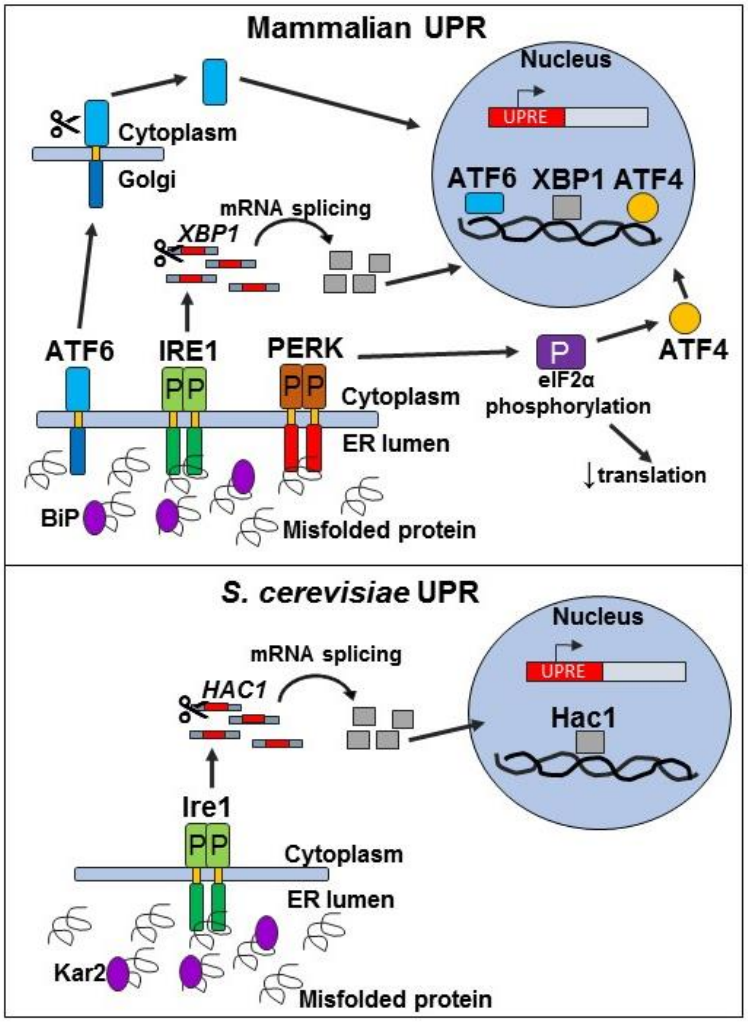

Figure 1.1: Mammalian UPR signaling pathway versus pathway in *S. cerevisiae*. While the mammalian UPR involves three signaling proteins, ATF6, IRE1, and PERK, the yeast UPR contains only Ire1. Both are activated by the presence of misfolded proteins in the ER, resulting in the activation of downstream signaling pathways. In the mammalian UPR, this is mediated by cleaved ATF6, XBP1, and eIF2α/ATF4. In the yeast UPR, the major effector is Hac1, an XBP1 homologue. The net effect of the UPR is an increase in ER chaperones, degradation machinery, ER lumen expansion, and decreased protein translation. Adapted from (Lajoie et al., 2014).

2005). When misfolded proteins are present, BiP dissociates to bind the misfolded proteins, causing derepression of the sensors. Upon activation, IRE1α monomers undergo autophosphorylation and oligomerization. The activated IRE1α complex splices the mRNA of its downstream target, X-Box-binding protein 1 (XBP-1), leading to the expression of a transcription factor, XBP1S. XBP1S translocates to the nucleus and controls the expression of a series of UPR-related genes through binding to an unfolded protein response element (UPRE) in the promoter sequence of these genes. This includes genes linked to protein folding, maturation and export, ER membrane synthesis for ER expansion, and ER-associated degradation (Calfon et al., 2002; Lee et al., 2003; Uemura et al., 2009). In addition, mammalian IRE1 can reduce misfolded protein burden by selectively cleaving ER-targeted mRNAs in a process known as Regulated IRE1-Dependent Decay (RIDD) (Hollien and Weissman, 2006; Hollien et al., 2009; Li et al., 2018). PERK activation leads to the phosphorylation of Eukaryotic Translation Initiation Factor 2α (eIF2α), which causes the formation of a stalled 43S ternary complex. This causes cell-wide translational attenuation, thereby reducing the protein folding burden in the ER lumen (Harding et al., 2000a, 2000b, 1999). Despite the translational attenuation, some proteins with internal ribosome entry sites (IRES) are actually upregulated; eIF2α phosphorylation upregulates both the chaperone BiP and Activating Transcription Factor-4 (ATF4), a basic leucine-zipper transcription factor essential for the transcription and translation of many UPR-associated genes that function in amino acid metabolism (Harding et al., 2000a) and redox homeostasis (Fels and Koumenis, 2006). After prolonged ER stress (and excessive UPR induction), this signaling pathway leads to the activation of CHOP, thereby activating apoptosis (Harding et al., 2000a; Marciniak et al., 2004). Finally, the ER-resident ATF6 traffics to the Golgi apparatus upon activation and is cleaved by specific proteases that release it as a transcription factor, ATF6(N). This transcription factor translocates to the nucleus and primarily increases the level of ER chaperones, which function to reduce ER stress by promoting proper folding and preventing aggregation (Shen et al., 2005, 2002; Wang et al., 2000). This includes chaperones such as BiP, PDI, GRP94, and calnexin (Harding et al., 2000b).

Unlike mammals, yeast have only one stress sensor: Ire1. When the yeast UPR is activated, the chaperone protein Kar2 (a BiP homologue) dissociates, allowing Ire1 to

oligomerize, autophosphorylate, and then activate downstream signaling pathways (Bertolotti et al., 2000; Kimata et al., 2003; Pincus et al., 2010). In *S. cerevisiae*, it does this by splicing *HAC1* mRNA, which can then be translated and act as a transcription factor, binding to UPRE sequences in gene promoters. Hac1 activates over 400 UPR target genes involved in responding to ER stress, such as chaperone proteins, ribosome biogenesis genes, ER-associated degradation (ERAD) effectors, and genes to expand the ER lumen (Aragón et al., 2009; Ma and Hendershot, 2004; Welihinda et al., 1999). Unlike mammalian cells, *S. cerevisiae* lack an mRNA-degrading mechanism like RIDD. However, in the fission yeast *Schizosaccharomyces pombe*, Ire1 reduces misfolded protein burden exclusively through RIDD and surprisingly lacks a *HAC1* homologue (Kimmig et al., 2012; Li et al., 2018). This separation of Ire1's functions allows each branch present in mammalian cells to be studied independently, though this thesis focuses on *S. cerevisiae* and Hac1p-mediated gene transcription. This pathway constitutes the fundamental basis of the UPR. Ire1 and Hac1, then, are key effectors of the *S. cerevisiae* UPR; the deletion or disruption of these two effectors can block/reduce UPR induction. This, in turn, can lead to lower tolerance for stress in the cell, and an inability to adapt to this stress, which can manifest as shortened lifespan or growth defects in yeast cells.

While much of the basic mechanism behind the UPR has been elucidated, not all aspects of it have been fully defined. The trigger for the switch between the UPR's adaptive (ER stress resolution) and maladaptive (apoptosis) processes, for example, is still unclear. The UPR has numerous links to apoptotic pathways through which this could occur, but there is no real consensus in the literature thus far. Some research points to the cause as activation of the mitochondrial apoptotic pathway through efflux of calcium ions from the ER lumen and uptake by the mitochondria, interaction of UPR sensors with Bcl-2 family proteins, or the activation of BH3-only proteins (Hetz, 2012; Rodriguez et al., 2012). It has been shown that IRE1's pro-survival signaling, wherein it degrades the apoptotic DR5 (Death Receptor 5) mRNA via RIDD to prevent apoptosis (Lu et al., 2014), initially increases during stress, but then attenuates after prolonged stress (Chawla et al., 2011; Rubio et al., 2011; Sun et al., 2016; Tay et al., 2014). Recently, this switch has been shown to occur through PERK and a downstream phosphatase, RPAP2; RPAP2 dephosphorylates IRE1, attenuating its activity and allowing DR5 and other pro-apoptotic

pathways to take over (Chang et al., 2018). The link between the UPR and these pro-apoptotic pathways, however, is not always straightforward, because many of the proteins shown to be involved in helping resolve ER stress are often the same ones indicated in stress-induced apoptosis. For example, IRE1 signaling, in addition to its normal adaptive response activity through XBP1/Hac1, has been shown to cause the activation of the downstream JNK (IRE1α–JUN N-terminal kinase) pathway via the TRAF2-ASK1 MAPK signaling pathway after a period of prolonged ER stress or UPR signaling. JNK in turn triggers apoptosis via inhibition of the pro-survival functions of Bcl-2 (Urano et al., 2000; Wei et al., 2008). IRE1 can also interact with the Bcl-2 family proteins BAX and BAK, which increase UPR signaling by activating the kinase/RNase activity of IRE1 to increase *XBP1/HAC1* mRNA splicing (Hetz, 2012; Hetz et al., 2006; Tabas and Ron, 2011). IRE1 also appears to cause the de-repression of Caspase-2 activation during ER stress, which leads to apoptosis (Upton et al., 2012). Caspases-3 and 7 have also been shown to cleave IRE1 in late stage ER stress, ending its pro-survival signaling but also paradoxically ending its pro-mitochondrial apoptotic interaction with BAX (Shemorry et al., 2019). Through PERK signaling, ATF4 can either assist with protein folding by increasing transcription of ER chaperones, or it can activate the pro-apoptotic proteins CHOP and GADD34 to cause apoptosis (Hetz, 2012; Sano and Reed, 2013).

Whether or not yeast undergo apoptosis is a controversial topic in the literature. While apoptosis is often beneficial for multicellular organisms (having been originally discovered in C. elegans (Kaczanowski, 2016)) it is difficult to see the benefit of programmed cell death for unicellular organisms. There is evidence that multicellular yeast colonies may use programmed cell death of those at the centre of the colony, supposedly in order to benefit younger, more metabolically active cells at the periphery (Váchová and Palková, 2005). There is also evidence of some evolutionarily conserved mammalian apoptotic mechanisms playing a role in yeast; chronologically aged yeast display apoptotic markers as well as caspase activation as they die (Herker et al., 2004), and expressing human anti-apoptotic Bcl-2 extends chronological lifespan (Longo et al., 1997). One study demonstrated that yeast with mutations in the cell cycle protein CDC48 show signs of apoptosis as well, including annexin V staining and DNA/chromatin fragmentation and condensation (Madeo et al., 1997). It is important to note, however,

that apoptosis-like morphological characteristics do not in and of themselves constitute apoptosis. There is still little evidence that yeast express their own apoptotic pathways, and many apoptotic gene/protein homologs found in yeast do not necessarily conduct the same activities as they do in mammalian cells (Aouacheria et al., 2013). For example, yeast metacaspases such as Yca1 are structurally similar to mammalian caspases but may or may not activate apoptotic programs in yeast (Madeo et al., 2002; Váchová and Palková, 2007; Wong et al., 2012). Yca1 leads to cell death after treatment with acetic acid or hydrogen peroxide (Madeo et al., 2002), but has also been shown to play other roles in the cell unrelated to cell death, such as regulation of cell cycle check points (Lee et al., 2008). It is therefore more likely that yeast are able to die by mechanisms other than spontaneous lysis, but do so in a functionally different way than mammalian apoptosis (Hardwick, 2018; Váchová and Palková, 2007).

Essentially, though many links between the UPR and apoptosis have been identified, the cellular decision-making process or threshold at which the apoptotic pathway is activated is still largely unknown. Some literature cites breakdown in ER-associated degradation as a contributing factor, while other research indicates that the amount of UPR-associated protein transcription taking place may cause the switch (Imai et al., 2000; Kaneko et al., 2002). It has also been shown that aging cells have a decreased tolerance for prolonged UPR signaling and may turn to the apoptotic pathway sooner, though this also has not been fully explained (Hussain and Ramaiah, 2007; Naidoo et al., 2008). The gaps in our current knowledge of the total reach of the UPR make the topic an interesting one for future research.

1.3 ER-Associated Degradation

ER-associated degradation is the process though which unfolded, misfolded, or otherwise damaged proteins are recognized, polyubiquitinated, retrotranslocated from the ER lumen to the cytosol, then degraded by proteasomes (Willer et al., 2008). Though ERAD is typically considered one overarching mechanism, it has several branches that can be activated separately. Depending on subcellular localization of the misfolded/unfolded protein, the process of ERAD is conducted by different machinery, and there are separate ERAD processes (ERAD-M at the membrane, ERAD-L at the lumen, ERAD-C in the

cytoplasm) with different effectors (Huyer et al., 2004; Meusser et al., 2005) (**Figure 1.2**). Specifically, the E3 ubiquitin ligases used to target these proteins for degradation (along with their associated protein complexes) are different, due to differentially recognizing degradation signals (degrons) dependent on their orientation relative to the ER membrane. The Hrd1 complex recognizes proteins with luminal or membrane-oriented degrons (ERAD-L or M), while the Doa10 complex recognizes proteins with cytoplasmic degrons (ERAD-C) (Carvalho et al., 2006; Rubenstein et al., 2012).

In regards to understanding ERAD's role in other cellular processes, it is imperative to differentiate which branch of ERAD is responsible for any results seen. Specifically, this has previously been done through the expression of ERAD substrates: proteins designed to misfold which can then overload and impair ERAD machinery. Many such proteins have been previously described. One example of this is carboxypeptidase Y* (or CPY*), which localizes to the ER lumen. The protein is mutated to misfold and can be overexpressed before it is dealt with by ERAD-L (Carvalho et al., 2010, 2006; Willer et al., 2008). Another example is Sec62-DEG1, which localizes to the ER membrane but is fused with a degron tag (DEG1) which targets it for degradation by Doa10, as opposed to the usual Hrd1. This appears to cause stalled retrotranslocation of the protein into the cytoplasm for degradation, leading to ER stress (Carvalho et al., 2010; Rubenstein et al., 2012). Essentially, though both proteins are ERAD substrates, their degradation is likely carried out through two different processes and can reveal different information about the cell's response to ER stress.

It is important to note, however, that despite the different effector proteins involved in the different branches of ERAD, it is unlikely that the two are completely unrelated. There is evidence to suggest that the pathways are more closely linked than previously thought, and could possibly compensate for one another if either pathway becomes impaired (Carvalho et al., 2006; Rubenstein et al., 2012; Willer et al., 2008). By understanding the mechanisms behind ERAD, and the specific functions that each branch of the pathway conducts, we could further our understanding of how these processes fail and the consequences of this. Because ERAD dysfunction has been implicated as a factor in the

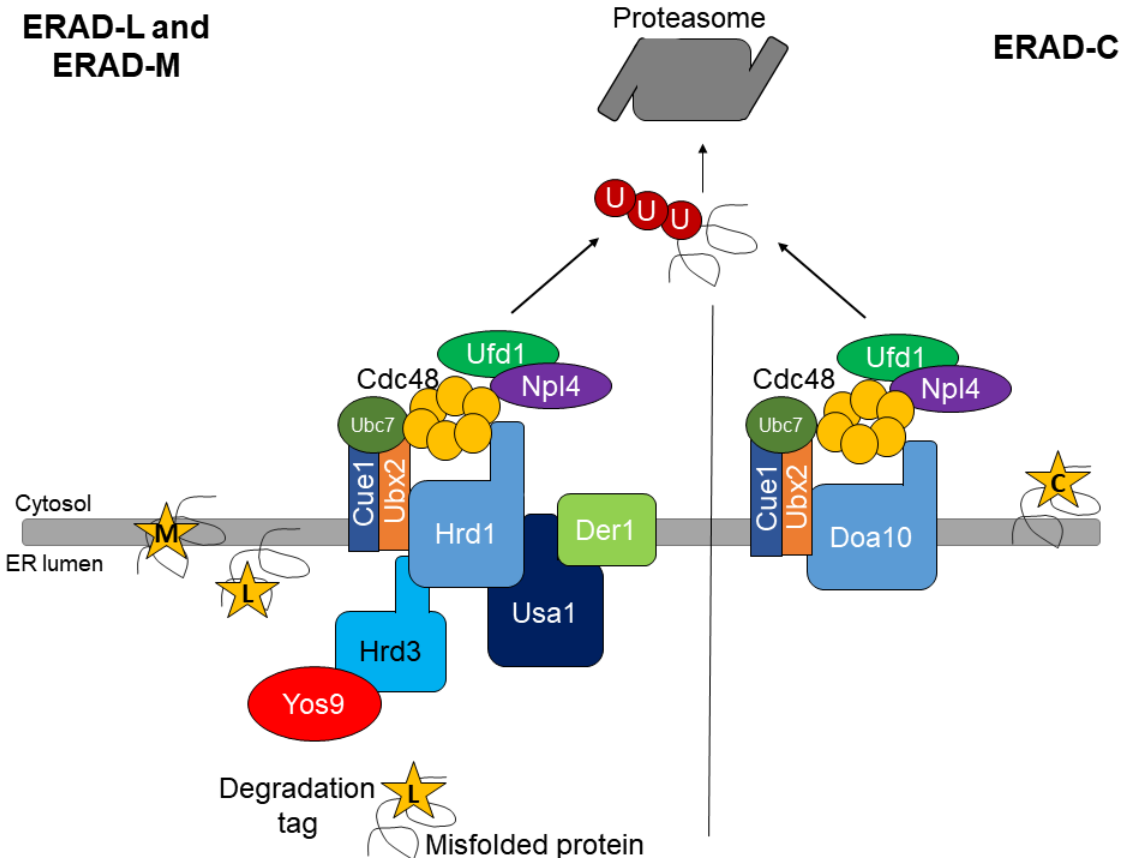

Figure 1.2: Schematic of ER-associated degradation in yeast. Proteins targeted for ER-associated degradation are tagged, then recognized by either the Doa10 or Hrd1 complex. Differential recognition depends on the location of the degradation tag: membrane and luminally tagged proteins are recognized by the Hrd1 complex, and cytosolic tags are recognized by the Doa10 complex. Tagged proteins are retrotranslocated from the ER lumen to the cytosol, polyubiquitinated, and degraded by the proteasome. Adapted from (Lemus and Goder, 2014).

breakdown of other cellular stress responses as well as several disease pathogeneses, this information could also be important for other fields of research.

1.4 Induction of ER stress

As previously mentioned, ER stress is the cellular state caused by various insults to the cell, including high misfolded protein burden in the ER, stalled protein folding or targeting, and a variety of other factors (Malhotra and Kaufman, 2007; Welihinda et al., 1999). ER stress leads to the activation of stress response pathways, such as the UPR, and increased ERAD (Friedlander et al., 2000; Liu and Chang, 2008). The resolution of ER stress is required for the cell to resume normal cellular functions and avoid maladaptive UPR effects (such as toxicity or apoptosis). ER stress can be induced by the accumulation of misfolded proteins, which can occur for a number of reasons, both naturally and artificially. Defective or impaired ERAD, for example, can lead to misfolded protein accumulation through inefficient removal of proteins targeted for degradation (Labunskyy et al., 2014; Ruggiano et al., 2014). Some disease phenotypes can also cause this; the expanded polyglutamine (polyQ) proteins which cause Huntington's disease can cause ER stress despite being cytoplasmic proteins. These abnormal proteins have been shown to sequester ERAD machinery, causing ER stress and UPR overinduction (Duennwald and Lindquist, 2008; Kouroku et al., 2002; Ravikumar et al., 2002; Scherzinger et al., 1999). The buildup of other protein plaques/aggregates (such as the amyloid-β plaques in Alzheimer's disease) can cause ER stress as well and result in sensitivity to other stressors (Lee et al., 2010). This is a common disease phenotype seen in neurodegenerative diseases. ER stress can also be induced artificially for research purposes. Tunicamycin treatment can induce ER stress specifically by blocking N-linked glycosylation (Kuo and Lampen, 1974), a protein modification initiated in the ER then completed in the Golgi apparatus. The drug blocks the first step of glycosylation, causing nascent glycoproteins to unfold and accumulate in the ER, leading to ER stress. Dithiothreitol (DTT) (Cleland, 1964) can also be used to induce ER stress, as it is a potent reducing agent that prevents the formation of disulphide bonds and causes denatured proteins to accumulate in the ER (Jämsä et al., 1994). Thapsigargin causes ER stress and UPR induction by depleting ER calcium stores, disrupting the protein folding

capabilities of calcium-dependent chaperone proteins such as BiP; it blocks the mammalian ER calcium pump, SERCA (sarco-endoplasmic reticulum Ca^{2+}-transport ATPase), and likely the yeast ortholog, Pmr1 (also a Ca^{2+} ATPase) (Földi et al., 2013; Lytton et al., 1991; Rudolph et al., 1989; Sehgal et al., 2017). Finally, as previously described, ER stress can be induced through the expression of ERAD substrates designed to misfold and/or stall the retrotranslocation step of ERAD (Carvalho et al., 2010; Lemus and Goder, 2014).

1.5 Lipid Modulation of ER Stress and the UPR

In addition to increased misfolded protein burden, ER stress can also be induced by environmental factors. Glucose deprivation (or caloric restriction) has been shown to mildly induce ER stress (Goldberg et al., 2009; Kaeberlein et al., 2005a). Lipid concentration and composition in cells or in media have also been shown to significantly impact ER stress and UPR induction (Promlek et al., 2011; Thibault et al., 2012).

There is evidence to suggest that the UPR sensors IRE1 and PERK are sensitive to perturbations of ER membrane lipid composition, even when lacking their luminal sensing domains (which typically sense misfolded/unfolded proteins) (Volmer et al., 2013). Other studies have also shown that the UPR is highly involved in responding to perturbation of lipid homeostasis (Thibault et al., 2012) and controls lipid synthesis and ER membrane proliferation in response to various cell stresses (Schuck et al., 2009). This suggests that the UPR is not limited to resolving ER stress from unfolded proteins but is also activated in response to changes in lipid saturation. This has been further validated in a *C. elegans* model of both disturbed proteostasis and lipid disequilibrium. It was found that compromised fatty acid distribution and reduced phosphotidylcholine production in the worms induced the UPR, even in the absence of proteostatic imbalance (Hou et al., 2014). Alterations in the yeast ER membrane lipid composition (not just saturation) have also been shown to induce the UPR in a mechanism independent of the presence of misfolded secretory proteins (Koh et al., 2018; Lajoie et al., 2012; Promlek et al., 2011), including by leading to the untimely removal of ER-resident transmembrane proteins, further exacerbating ER stress (Shyu et al., 2019). These findings indicate that multiple modes of UPR activation may exist. Similar activation of UPR sensors has also

been reported in mammals, indicating that this process is evolutionarily conserved (Volmer et al., 2013).

Of particular interest is one lipid which has been shown to be significant in ER stress/lipid equilibrium: inositol. Inositol has been shown to be a key regulator of cell stress responses, both in yeast and in higher models, so supplementing inositol has the potential to reduce ER stress (Lajoie et al., 2012; White et al., 1991). Inositol has also been shown to downregulate key phospholipid biogenesis genes, such as *INO1*, *OPI1/3*, and *CHO1/3* (Henry et al., 2014). This previous research could have implications in terms of modulating UPR induction and ER stress caused by other stressors unrelated to lipids, such as misfolded proteins. By stabilizing/supplementing the lipid balance of cells with misfolded protein stress (either by adding lipid to the media, or by inducing endogenous lipid production), the overall induction of the UPR could be reduced, possibly rescuing cells from the toxic effects of UPR overinduction seen in several diseases (Hou et al., 2014; Thibault et al., 2012).

There are numerous gaps in our understanding of ER stress, the UPR, and how the processes relate. While some possible mechanisms have been proposed to explain the interaction of lipids with the UPR, for example, it is difficult to determine which (if any) of these is correct. Early studies on the UPR identified that deletion of *IRE1* removes yeast inositol auxotrophy (hence the name “Inositol Requiring Enzyme”), and it was originally proposed that phospholipid synthesis and the UPR may be linked through the expansion of the ER membrane (to accommodate increased protein folding burden) as part of the UPR signaling pathway (Nunnari and Walter, 1996). Though the role of Ire1 has since been clarified, the link between inositol and ER membrane expansion is still likely and has been supported by the literature (Cox et al., 1997; Henry et al., 2014; Nikawa and Yamashita, 1992). Recently, Ire1’s activation by lipid bilayer stress has been shown to occur separately from its activation by misfolded proteins, and has also been shown to activate an altered transcriptional program (Ho et al., 2019; Koh et al., 2018). The exact mechanism through which this occurs, however, and which other UPR or lipid synthesis proteins or processes are involved have yet to be fully identified, offering an interesting new topic to be studied for UPR-related diseases.

1.6 The Unfolded Protein Response in Disease

The unfolded protein response has been implicated in several different diseases, from diabetes to cancer. Its role in the development of these diseases is far from consistent, however, and the diseases in which it is involved vary widely as well.

Several studies have indicated that the UPR plays a role in diabetes. In particular, the PERK/eIF2α branch of the UPR appears important for glucose homeostasis. PERK-mediated phosphorylation of eIF2α is essential for translational attenuation during ER stress. It has been shown that mice expressing a form of eIF2α that cannot be phosphorylated (and thus cannot be regulated by PERK during the UPR) showed defects related to diabetes. The mice had disrupted gluconeogenesis in the liver and pancreatic β cell insufficiency causing decreased insulin production (Scheuner et al., 2001). This suggests that regulation of eIF2α by PERK and the UPR is required for proper glucose homeostasis. $PERK^{-/-}$ mice have also been shown to develop progressive pancreatic insufficiency and other diabetes-like symptoms postnatally, along with increased IRE1 activity and increased apoptosis in pancreatic islet cells (Harding et al., 2001). Similarly, Wolcott-Rallison syndrome, an autosomal recessive neonatal- or early infancy-onset form of insulin-dependent diabetes, is caused by mutations in eIF2α-kinase 3 (Delépine et al., 2000), further suggesting a role for the PERK branch of the UPR in development of diabetes. Obesity is a common risk factor for type 2 diabetes, and a study by Özcan et al. demonstrated that obese mice had increased overall levels of ER stress and UPR signaling, demonstrated through increased PERK and eIF2α phosphorylation, which led to decreased insulin action in liver cells. They also showed that mice with either decreased IRE1 or XBP1 levels had impaired glucose metabolism and decreased sensitivity to insulin, as well as notable increases in ER stress (Ozcan et al., 2004). Similarly, in another study, mice lacking XBP1 only in pancreatic β cells showed hyperglycemia and islet cell loss, as well as hyperactivation of IRE1 and ER stress (Lee et al., 2011). An excess of XBP1, however, has also been shown to be toxic; XBP1 overexpression appears to cause impaired insulin secretion in response to glucose, as well as increased β cell apoptosis (Allagnat et al., 2010).

Another area of UPR research examines the connection between the UPR, heart disease, and atherosclerosis. As discussed, both obesity and lipid metabolism are closely linked to UPR function, and both play a role in heart disease. Increased UPR signaling can lead to increased lipid biogenesis, and declining UPR efficiency in resolving stress can cause excessive and prolonged UPR activation. This can lead to impaired glucose metabolism, insulin resistance, hyperlipidemia, and weight gain, potentially causing further ER stress (Hou and Taubert, 2014; Ozcan et al., 2004; Szegezdi et al., 2006). It has also been shown that insulin resistance in macrophages can lead to increased ER stress and higher sensitivity to stress-induced apoptosis, including stressors such as free cholesterol loading, as well as impaired phagocytic capacity. This process has been implicated in the progression of atherosclerotic lesions; ER stress is a hallmark of advanced atherosclerosis. As macrophages experience increased ER stress and UPR signaling, they are less able to tolerate cholesterol loading and clearance of plaques, leading to apoptosis and further plaque progression (Feng et al., 2003; Han et al., 2006). This may occur through maladaptive signaling of the UPR; it has been shown that $CHOP^{-/-}$ mice fed a high fat diet developed similar plasma lipoprotein levels as their $CHOP^{+/+}$ counterparts, but the size of both the atherosclerotic lesions and necrotic cores of the plaques was significantly reduced when CHOP activity was eliminated (Han et al., 2006). Together, these data suggest a role for the UPR and ER stress in heart disease (through increased lipidemia/decreased glucose tolerance) and atherosclerosis.

The UPR has also been identified as essential in cancer proliferation and survival. Solid tumors are characterized by hypoxia and their ability to survive despite this. Their resistance to hypoxia appears to be conferred, at least in part, by activation of the UPR, and these resistant cells have been shown to also become resistant to stress induced by radiation therapy (Fels and Koumenis, 2006; Rouschop et al., 2013). This appears to occur through the PERK/eIF2α branch of the UPR; it has been shown that inhibition of eIF2α in cancer cell lines significantly reduces survival during severe hypoxia (both chronic and intermittent) but does not affect tumor size or cell numbers. The same study showed that transient eIF2α inhibition also increased response to radiation treatment (Rouschop et al., 2013). Another study showed that PERK-deficient cells accumulated reactive oxygen species (ROS) during hypoxia, and this caused oxidative DNA damage

and reduced cell growth (Bobrovnikova-Marjon et al., 2010). The IRE1 pathway may also play a role; one study demonstrated that IRE1 may play a protective role against malignant glioma. IRE1 inhibition was shown to increase cancer cell invasiveness and survival while decreasing tumor vasculature development, which is a hallmark of the angiogenic to invasive switch that many aggressive cancers undergo (Auf et al., 2010). XBP1 was also found to be overexpressed in colon cancer cells compared to normal colon cells, indicating a general increase in UPR signaling in cancer cells (Fujimoto et al., 2007). mRNA levels of vascular endothelial growth factor A (VEGFA), an important factor in angiogenesis shown to contribute to tumor growth and survival, are also tightly regulated by the UPR. All three branches (ATF6, PERK, and IRE1) have been shown to upregulate VEGFA in response to ER stress, which may play a role in tumors' ability to grow despite prolonged stress (Ghosh et al., 2010).

Finally, ER stress and the UPR have been linked to the development and progression of neurodegenerative disease. Many neurodegenerative diseases are linked to the accumulation of misfolded proteins over time. In Alzheimer's disease, for example, tau neurofibrillary tangles and amyloid-β plaques accumulate in neurons and lead to neurodegeneration. Studies have shown that cells with these protein aggregates have high UPR induction as identified through high levels of phosphorylated eIF2α, PERK, and IRE1 (Hoozemans et al., 2009, 2005). This has been identified in early stages of protein accumulation and linked to later stage neurodegeneration, suggesting an early role for the UPR that may later become maladaptive (Hoozemans et al., 2009). In Parkinson's disease, misfolded proteins accumulate in the substantia nigra region of the brain, leading to loss of dopaminergic neurons in this region. Similar to the studies performed on Alzheimer's disease, it has been shown that the UPR is highly activated in these areas and that this UPR activation may be causally linked to the neurodegeneration seen in this disease (Hoozemans et al., 2007). Similar findings have also been reported in spinal cord tissue from patients with sporadic Amyotrophic Lateral Sclerosis (ALS) (Atkin et al., 2008). Huntington's disease is another neurodegenerative disease which is characterized by the accumulation of misfolded huntingtin (Htt) protein, which undergoes abnormal expansion of a segment of polyglutamine (polyQ) repeats. Longer polyQ tracts are associated with earlier onset and more severe symptoms (Macdonald, 1993).

Interestingly, longer polyQ tracts are also more prone to aggregation and are associated with a higher degree of UPR induction but a lower degree of HSR induction (Chafekar and Duennwald, 2012; Martindale et al., 1998). It has also been shown that ER stress and impaired ERAD due to sequestration of ERAD machinery occur early in Huntington's pathogenesis (Duennwald and Lindquist, 2008; Lajoie and Snapp, 2011). Taken together, these results suggest that dysregulation of ER stress response pathways can lead to maladaptive UPR signaling over time, which in turn may lead to neurodegeneration through UPR-mediated apoptosis.

The UPR and its effectors appear to play a wide variety of roles in producing or contributing to disease; understanding how the UPR and ER stress impact different systems is crucial to understanding how they function and change depending on different circumstances.

1.7 Aging

Aging has been shown to modulate some of the factors leading to ER stress. It is an important modifier of the proteostasis network, meaning that aging cells may have altered capacity to properly carry out protein transcription, translation, folding, and degradation (Brown and Naidoo, 2012; Naidoo, 2009). Aging cells have been shown to have decreased levels of a number of ER proteins, including protein chaperones (such as PDI, BiP, etc.) which normally supervise and ensure proper protein folding, and assist in targeting misfolded proteins for degradation (Hussain and Ramaiah, 2007; Paz Gavilán et al., 2006). This usually prevents the accumulation and aggregation of misfolded proteins and prevents them from having toxic effects on the cell. In addition, the limited chaperones that are still present in the aging ER appear to be impaired; this is possibly due to the progressive oxidation of these chaperones over time, leading to decreased function. Both BiP ATPase activity and PDI enzymatic function have been shown to be significantly decreased in aged mouse livers (Nuss et al., 2008), and similar results have been seen in a number of other models as well, such as aged mouse cerebral cortex (Naidoo et al., 2008). Other components of UPR signaling have also shown to be reduced during aging. PERK mRNA, for example, has been shown to be reduced in aged rat hippocampi, indicating less efficient UPR signaling (Paz Gavilán et al., 2006). Total BiP

protein levels are also reduced in aging models of both rats and mice (Hussain and Ramaiah, 2007; Naidoo et al., 2008).

Aging also appears to alter the threshold at which the UPR switches from the adaptive pathway to the apoptotic pathway, which is perhaps related to the changes to proteostasis previously mentioned. When PERK signaling is decreased during aging, for example, there is evidence of an increase in GADD34 expression, which helps remove the translational block that occurs through PERK phosphorylating eIF2α. This allows the expression of pro-apoptotic proteins, such as CHOP (Brown and Naidoo, 2012). CHOP has been shown to be increased with stress during aging (Hussain and Ramaiah, 2007; Naidoo et al., 2008), as has caspase-12, but not during stress in younger cells (Paz Gavilán et al., 2006). The apoptotic protein JNK (which is activated by IRE1 during prolonged UPR signaling) is also upregulated during aging, as are JNK kinases that phosphorylate other apoptotic transcription factors such as ATF-2 and c-Jun (Brown and Naidoo, 2012; Hussain and Ramaiah, 2007). This aging-related decrease in adaptive UPR signaling and increase in apoptotic signaling may account for the apparent sensitivity of aged cells to ER stress, and the increased rate of cell death amongst stressed cells when aged.

Aging is a common risk factor for a number of diseases, including cancer, heart disease, and diabetes, a number of which have links to UPR function as described in the previous section (Brown and Naidoo, 2012). While not all aging-related diseases are directly linked to breakdown of UPR signaling, this breakdown may still contribute to disease pathogeneses. For example, type 2 diabetes is known to develop more frequently due to both obesity and aging, with the two factors often coexisting in patients (Ozcan et al., 2004). As aging has also been linked to the same reduction of UPR components associated with diabetes and insulin resistance, these results suggest that aging-related UPR defects may be linked to these diseases as well. Obesity and insulin resistance are also linked to heart disease and atherosclerosis, both of which increase in prevalence with age and have also been linked to ER stress and the UPR (Han et al., 2006). Finally, numerous neurodegenerative diseases have been linked to the UPR and ER stress, and most have an aging component. Huntington’s disease, Parkinson’s disease, and

Alzheimer's disease have been clearly and repeatedly linked to UPR dysfunction which increases with age, thus increasing disease severity (Carroll et al., 2013; Vidal and Hetz, 2012). Though the three diseases have different causative genes, they all share misfolded protein accumulation and aggregation as part of their pathology, leading to impaired proteostasis and ER stress responses, and then cellular toxicity. Increased ER stress in neuronal cells as a result of impaired ERAD and UPR during aging also sensitizes them to apoptosis, leading to neurodegeneration (Lajoie and Snapp, 2011; Lee et al., 2010; Paz Gavilán et al., 2006). Aging is an important factor in understanding how cells cope with stress, and how these coping mechanisms change with time and different cellular conditions.

1.8 Yeast Model of ER stress and Aging

The research presented here aims to study the effect of ER stress responses on aging with a budding yeast model. The model is valid for this research since *Saccharomyces cerevisiae* share many of the same ER stress response pathways as mammalian cells, but the molecular pathway is simplified and therefore easier to manipulate and study (Mori, 2009). More specifically, only one of the three mammalian UPR sensors (IRE1) is present in yeast, but the signaling pathway itself is otherwise very similar. The UPR and ER stress disease mechanisms (in age-related diseases, including Huntington's disease, Parkinson's disease, etc.) identified in yeast have also been confirmed in animal models, adding further validity to their use as a model for aging (Gitler, 2008). Yeast offers the possibility to study two aging paradigms: chronological and replicative aging (**Figure 1.3**). Replicative aging is a measure of the number of cell divisions a yeast cell has undergone, whereas chronological aging measures actual time post-mitotic cells spend growing in media without the addition of nutrients (Steffen et al., 2009). Here, we are studying ER stress and aging-related cellular dysfunction over the course of chronological aging (as opposed to replicative) because it mimics many of the disease mechanisms and cell responses seen in aging mammalian cells, and because the role of the UPR in chronological aging is not well known (Duennwald, 2013; Harris et al., 2001; Longo et al., 2012). Yeast cells with a compromised UPR do not have a reduced replicative lifespan, for example; replicative aging would, therefore, not be affected by

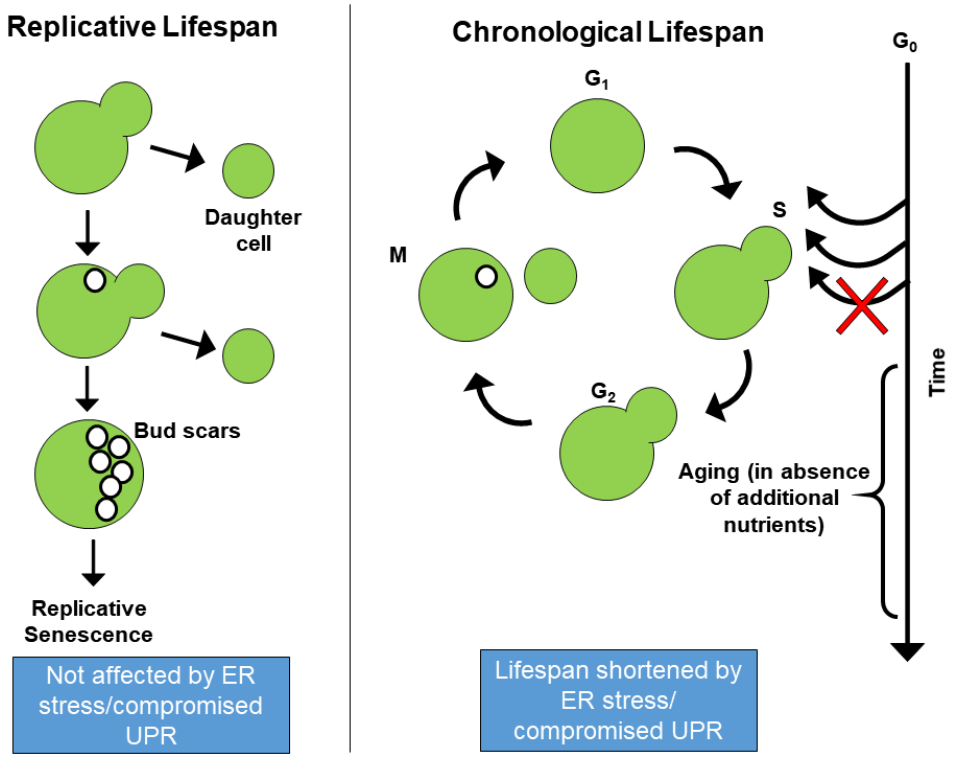

Figure 1.3: Yeast models of aging. Yeast have two forms of aging: chronological and replicative. Replicative lifespan is the number of times a cell divides before reaching replicative senescence, and chronological lifespan is the amount of time a cell survives in the absence of additional nutrients after exiting mitosis. Chronological aging is most often used to study ER stress responses because it is shortened by ER stress or a compromised UPR, whereas replicative lifespan is not.

altering or rescuing the UPR or other cell stress mechanisms. Chronological aging is also better suited for studying non-dividing cells such as neurons, which have been shown to be particularly sensitive to perturbed protein folding and ER stress responses (these disrupted mechanisms have also been clearly linked to several different neurodegenerative diseases) (Labunskyy et al., 2014).

The yeast model of chronological aging has been used extensively over the years, adding further validity to its use here. Yeast research also often reflects systems present in mammalian cells, and many major discoveries (in the field of aging and otherwise) were first identified in yeast. Caloric restriction, for example, is a well-established method to extend lifespan in mammalian cells and whole-animal systems, and was first discovered in yeast (Colman et al., 2009; Jiang et al., 2000). Another important finding in yeast was the role of sirtuins in aging. Sirtuins are a family of NAD-dependent deacetylases that were initially discovered to play a role in replicative lifespan in yeast; specifically, these proteins were found to regulate the recombination of ribosomal DNA (Gottlieb and Esposito, 1989). It was later found that sirtuins reduce the production of extrachromosomal rDNA circles (ERCs), which were in turn found to be elevated during yeast aging (Sinclair and Guarente, 1997). They also appeared to normally be responsible for silencing at telomeres, and the mutation of Sir4 was found to cause its relocation to the nucleolus which extended lifespan, potentially by stabilizing rDNA/preventing the formation of ERCs (Kaeberlein et al., 1999; Kennedy et al., 1997, 1995). Sirtuin homologues have since been found in *Drosophila melanogaster* and *C. elegans*, as well as linked to lifespan extension through calorie restriction (Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). Seven mammalian homologues have also been identified and associated with longevity and other hallmarks of aging (Mostoslavsky et al., 2006; Satoh and Imai, 2014). Finally, the TOR (target of rapamycin) pathway was also initially identified in yeast when it was found that rapamycin treatment caused cell cycle arrest through binding to specific proteins (TOR1 and 2 in yeast) (Heitman et al., 1991). TOR is a protein kinase involved in nutrient sensing, cell cycle regulation, and regulation of autophagy, and has since been identified as highly evolutionarily conserved (though typically existing as only one homologue, not two as in yeast) (Martin and Hall, 2005). Later yeast studies have shown that TOR inhibition (through rapamycin treatment

or otherwise) extends both replicative and chronological lifespan and increases stress resistance, possibly through induction of autophagy (Kaeberlein et al., 2005b; Powers et al., 2006). These results have since been duplicated in invertebrate and mammalian models (Kapahi et al., 2004; Vellai et al., 2003; Wilkinson et al., 2012).

One major pitfall of the yeast chronological aging model is that the current methods available to study this form of aging rely primarily on the ability of the aging cells to regrow. These methods include spot assays, in which serial dilutions of the cultures are plated and assessed for growth defects, or colony forming unit assays, in which samples of the culture are re-plated on solid media to determine how many colonies grow. This is a more quantitative method, as is a bioscreen/growth curve assay, in which cells are added to new media and while the optical density of the culture is measured over time to assess regrowth and infer viability. Alternatively, competition-based growth assays can be used to assess relative fitness, and when combined with barcode-tagged gene deletion libraries (wherein each strain contains a unique DNA "barcode" which can be used to assess its relative proportion in a population), this can be a high throughput method for measuring the effect of a wide range of specific genes on growth in parallel over the course of aging (Matecic et al., 2010; Pierce et al., 2007). While certainly effective, regrowth assays are not necessarily a direct measure of cell viability in aging cultures. Cells can also be directly tested for viability using dyes specific to either live or dead cells, which are often detected through fluorescence. Fluorescent microscopy is one method to measure viability staining, and while accurate, it is not high-content and may be difficult when dealing with a large number of samples or experimental conditions. Alternatively, fluorescence-activated cell sorting (FACS) is a high-throughput method to measure relative levels of fluorescence but can be expensive and is not accessible to all labs. These methods are effective but could certainly be improved upon. Despite this, yeast viability assays remain an excellent tool for studying functional genomics and the influence of numerous signaling pathways on growth and survival. In addition to ER stress, yeast also offer the opportunity to study related cell stress response pathways and their effect on chronological aging, as described in the following sections.

1.9 Cell Wall Integrity Pathways

Cell wall integrity (CWI) is an important stress response pathway that has been repeatedly linked with ER stress and the UPR. The yeast cell wall is a complex protein and carbohydrate structure that is important for resistance to changes in osmotic pressure and polarized cell division via bud formation, among other functions (Levin, 2005). The biosynthesis and maintenance of this cell wall is dependent upon proper protein folding and secretion (as it is an extracellular organelle) (Krysan, 2009); it is therefore unsurprising that its integrity is dependent upon proper secretory pathway and ER function as well. CWI signaling occurs primarily through a series of plasma membrane-bound stress sensors (such as Wsc1-3 and Mid2) which activate Rho1, an amplifying cascade of MAP kinase proteins (including Pkc1 and Slt2/Mpk1), and transcription factors (such as Rlm1 and Swi4/6) which induce the transcription of cell wall components (**Figure 1.4**) (Bonilla and Cunningham, 2003; Nunez et al., 2008; Scrimale et al., 2009; Verna et al., 1997). Previous research indicates that cell wall stress activates the UPR similarly to ER stress (Krysan, 2009; Scrimale et al., 2009) in that cell wall-perturbing agents have been demonstrated to increase signaling of genes containing UPRE sequences (Scrimale et al., 2009). Conversely, ER stress has been shown to activate CWI signaling pathways (Bonilla and Cunningham, 2003; Krysan, 2009; Levin, 2011). This signaling pathway is also activated by several stressors that only indirectly affect the cell wall, including oxidative stress, DNA damage, and heat stress (Sanz et al., 2017). Additionally, cell wall stress has been shown to affect the transcription a number of other genes, including upregulation of general cell stress genes encoding heat shock proteins (Hsp12, Hsp42) and those containing generalized stress response promoter sequences (STRE) (Arroyo et al., 2009; Boorsma et al., 2004). It also increases transcription of energy storage/metabolism and osmosensing genes, while decreasing expression of genes required for ribosomal biogenesis and rRNA transcription and processing (associated with a decrease in growth) (Boorsma et al., 2004). Cell wall stress, then, may activate a global stress response and make cells more prepared to withstand other forms of stress. Cell wall stress can be induced in a variety of ways. Deletion of cell wall biogenesis/maintenance genes, for example, causes cell wall stress. It can also be artificially induced using cell wall-disrupting agents, such as caspofungin (an anti-fungal

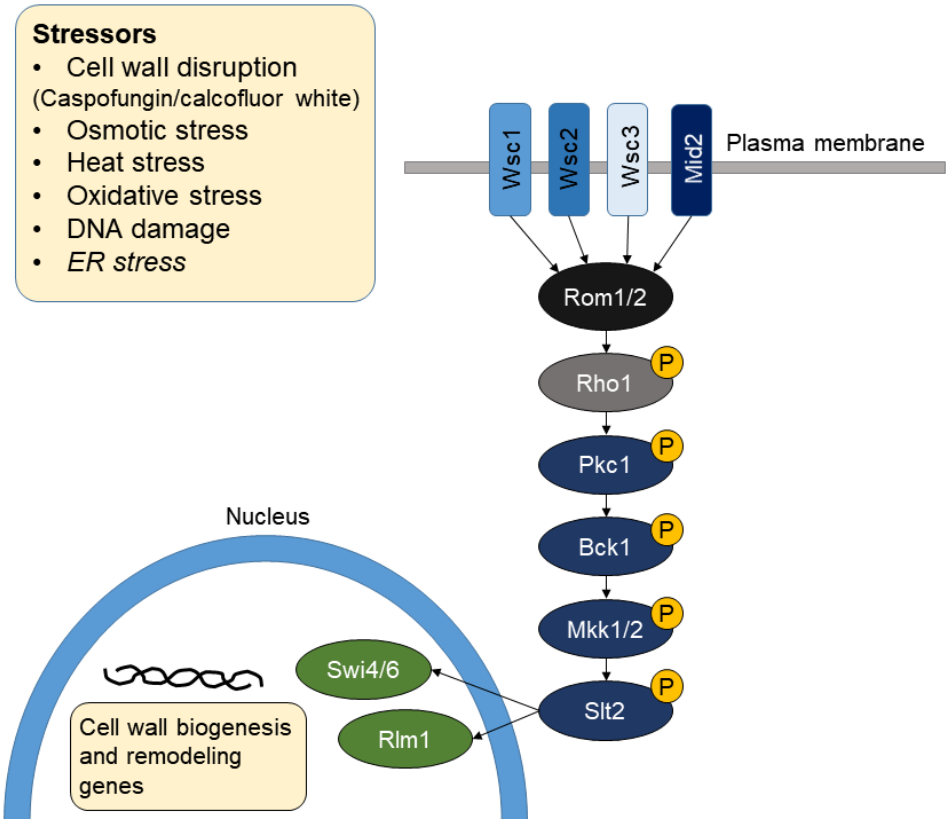

Figure 1.4 Cell Wall Integrity Pathway signaling. Activation of CWI signaling by various stressors is initiated by stress sensing proteins, Wsc1-3 and Mid2. These sensors interact with Rom1/2, which activates GTPase Rho1. Rho1 begins a MAP kinase signaling cascade, ending with Slt2. Slt2 activates Swi4/6 and Rlm1, transcription factors which upregulate genes involved in cell wall biogenesis, maintenance, and remodeling. Adapted from (Kock et al., 2015).

which inhibits β-1,3-glucan synthase (Walker et al., 2015)) and calcofluor white (which binds to chitin (Heilmann et al., 2013)).

1.10 Chemical Chaperones and Pharmacological Interventions

Numerous attempts have been made to find new ways to modulate ER stress and cellular stress responses. One approach could be to address the root cause of ER stress: misfolded proteins. One could reduce ER stress and resultant stress response activation by reducing the misfolded protein burden within the ER lumen, either artificially or through the cell's own mechanisms (such as ER-associated degradation or autophagic removal of misfolded protein-containing sections of the ER). Another approach could be to modulate stress signaling independent of misfolded protein burden. Decreasing maladaptive or excessive UPR signaling despite the buildup of misfolded Htt protein in HD, for example, could reduce neuronal stress sensitivity and apoptosis/cell death, and thereby reduce resultant neurodegeneration. The goal, then, would be to discover or repurpose small molecules or drugs that can modulate ER stress through either of these approaches. Chemical chaperones are one promising group of small molecules that may be able to achieve this.

Chemical chaperones are generally low molecular weight compounds known to stabilize protein conformation, improve ER folding capacity, and facilitate the trafficking of mutant proteins (Ozcan et al., 2006; Welch and Brown, 1996). Some examples tested in the literature include 4-phenylbutyric acid (4-PBA), trimethylamine N-oxide dihydrate (TMAO), dimethyl sulfoxide (DMSO), glycerol, as well as some bile acids and their derivatives (such as ursodeoxycholic acid (UDCA) and its taurine conjugate, tauroursodeoxycholic acid (TUDCA)) (Berger and Haller, 2011; Upagupta et al., 2017; Welch and Brown, 1996). Many of these have had very promising results. In studies of mutant protein trafficking, 4-PBA, glycerol, deuterated water (D_2O), and TMAO were shown to improve the trafficking and temperature sensitivity of the cystic fibrosis-associated mutant ion transporter CFTR ΔF508, which has folding defects resulting in its degradation via ERAD before it can be trafficked to the cell surface (Brown et al., 1996; Sato et al., 1996). 4-PBA has also been shown to improve the secretion of mutant α1-ATZ protein in α1-antitrypsin deficiency, the most common genetic cause of liver disease

in children (Burrows et al., 2000), and can also rescue the trafficking of the coagulation protein PCA267T which can become retained in the ER or the Golgi (Chollet et al., 2015). Both TUDCA and 4-PBA were able to alleviate ER stress in a mouse model of type 2 diabetes; these compounds also were shown to reduce hyperinsulinemia and restore normal glucose tolerance (Ozcan et al., 2006). In a study of prion proteins, DMSO, TMAO, and glycerol were all shown to reduce the formation of the scrapie prion protein from its wild-type form (though they did not clear existing prion species) (Tatzelt et al., 1996). DMSO, TMAO, and glycerol have also been shown to restore the trafficking of aquaporin-2 (AQP2) mutants in nephrogenic diabetes insipidus, restoring normal water permeability of cells (Tamarappoo and Verkman, 1998).

The mechanisms by which these chemical chaperones reduce ER stress is not well known and may vary between compounds. Glycerol, for example, has been proposed to work due to its exclusion from many domains of nascent polypeptides; it could increase hydrophobic effects within proteins, which in turn respond by folding more tightly and stabilizing, making them less sensitive to heat or other disruptions (Brown et al., 1996). This was proposed as an explanation for glycerol's aforementioned effect on mutant CFTR trafficking; the phenylalanine deletion in the ΔF508 mutant could cause a loss of a hydrophobic interaction, and glycerol restores this. Glycerol was also found to reduce cell growth and protein synthesis; slowing down this process may also allow mutant proteins more time to fold properly (Brown et al., 1996). Upagupta et al. characterized the chemical and ER stress-reducing properties of several proposed chemical chaperones and found that those with a localized hydrophilic and polar end followed by a long hydrophobic chain (such as 4-PBA and docosahexaenoic acid, DHA) were generally the most effective at reducing ER stress (Upagupta et al., 2017). They measured protein aggregation during thapsigargin-induced ER stress; the most dramatic reduction in protein aggregation was seen in DHA, TUDCA, and 4-PBA, while trehalose and glycerol showed the least. DHA's effect was more potent than that of 4-PBA, which the authors proposed could be because its hydrophobic chain is longer than that of 4-PBA. It should be noted, however, that the vehicle control for these experiments was DMSO, which may have chemical chaperone effects of its own.

While chemical chaperones are generally able to reduce ER stress, they do so by reducing misfolded protein burden directly. It is therefore important to differentiate between compounds that act as chemical chaperones and those that reduce ER stress independently of protein misfolding. Some proposed chemical chaperones that have potent effects on ER stress have not been proven to directly affect protein folding, stability, or trafficking, and may have therefore been mischaracterized. One such example is TUDCA, which will be discussed in more depth in following sections.

In addition to chemical chaperones, there are also many other pharmacological modulators of ER stress targeting all three branches of the UPR. IRE1, for example, has been targeted in several ways. Small molecule inhibitors of its endoribonuclease domain (responsible for cleavage of *HAC1* mRNA) as well as its kinase domain (which catalyzes the trans-autophosphorylation step that activates IRE1) have been developed for various in vitro and disease models (Papandreou et al., 2011; Sato et al., 2017; Volkmann et al., 2011; Zhao et al., 2018). Allosteric inhibition of IRE1 (through prevention of the oligomerization step of its activation) has also been successful in reducing UPR activation, both to prevent unfavourable UPR-mediated apoptosis and to increase sensitivity to chemotherapeutics (Cross et al., 2012; Ghosh et al., 2014; Waller et al., 2016; Wang et al., 2012). Similarly, inhibition of the PERK pathway is another attractive target. PERK itself has been targeted for inhibition, as have its downstream factors, eIF2α and ATF4. PERK inhibition was found to reduce pancreatic cancer growth (Axten et al., 2012). Inhibition of dephosphorylation of eIF2α (using a phosphatase inhibitor) was shown to be protective against ER stress, and also reduced replication of Herpes Simplex Virus (Boyce et al., 2005). Inhibition of ATF4 production using ISRIB (Integrated Stress Response Inhibitor) blocked PERK-specific UPR activation and Integrated Stress Response (ISR) activation; it also rendered cells more sensitive to ER stress and improved memory consolidation in rodents (Sidrauski et al., 2015, 2013). Finally, the third branch of the UPR, ATF6, has been targeted for both inhibition and activation. It was first inhibited by preventing its translocation to the Golgi and subsequent cleavage, thereby preventing its activation. This caused decreased stress adaptation and increased sensitivity to prolonged ER stress (Gallagher and Walter, 2016; Gallagher et al., 2016). More recently, activation of ATF6 has been shown to be beneficial in multiple disease

models, including ischemia/reperfusion injuries of the heart, kidneys, and brain, and liver damage in dysregulated hepatic proteostasis (Blackwood et al., 2019). The safety profiles and off-target effects of the numerous pharmacological interventions listed here can vary, and new ways of modulating ER stress for different purposes are still needed.

1.11 TUDCA and ER stress

Tauroursodeoxycholic acid (TUDCA) is a taurine-conjugated bile acid produced by intestinal bacteria. While it is produced only in small amounts in humans, it is the primary bile acid produced in Asian and North American black bears (Boatright et al., 2009). Bear bile and bile extract have been used in traditional Chinese medicine for thousands of years to treat a variety of conditions, but more recently TUDCA has been identified as a potential cytoprotective treatment in conditions such as retinal degeneration (Drack et al., 2012), Alzheimer's disease (Nunes et al., 2012), spinal cord injury (Kim et al., 2018), Huntington's disease (Keene et al., 2002), and many others (Vang et al., 2014). Despite promising research in all of these fields, its exact mechanism of action is still unclear; it affects a variety of signaling pathways and has numerous targets (including inhibition of apoptotic effector proteins such as Bax, p53, caspases, etc.) (Vang et al., 2014; Xie et al., 2002). As previously mentioned, TUDCA is hypothesized by some groups to act as a chemical chaperone in the context of UPR and ER stress signaling by stabilizing protein conformation, thereby improving the folding capacity of the ER (Omura et al., 2013; Ozcan et al., 2006). It has indeed been repeatedly shown to reduce ER stress and improve stress response signaling, but thus far there is no evidence to support its direct role in protein folding or trafficking.

TUDCA's chemical structure contains four aromatic rings, similar to a steroid hormone. It is also amphiphilic with both hydrophobic and hydrophilic structures and is more hydrophilic than its unconjugated form (UDCA) ("Tauroursodeoxycholic acid | C26H45NO6S - PubChem," n.d.). While fewer than 10% of bile acids produced in the human body reach the systemic circulation (Mertens et al., 2017), many have been detected in plasma and cerebrospinal fluid, and bile acid receptors and transporters exist throughout the body (Daruich et al., 2019; Reinicke et al., 2018). For TUDCA specifically, these receptors include glucocorticoid/mineralcorticoid receptors (liver,

brain, and retina), takeda G-protein coupled receptor 5 (TGR5) (liver, intestine, brain, eye, lung, spleen, monocytes), sphingosine 1-phosphate receptor 2 (S1PR2) (liver, brain, heart, lung, eye), and α5β1 integrin (liver, brain, retina) (Daruich et al., 2019). Conjugated bile acid transporters (which likely transport TUDCA) include apical sodium-dependent bile acid transporter (ASBT) (intestine, cholangiocytes, brain), sodium taurocholate co-transporting polypeptide (NTCP) (liver, brain), organic anion-transporting polypeptide (OATP) (liver, intestine, brain, retina), multidrug resistant proteins (MRPs) 2-4 (liver, brain, retina), and the bile salt export pump (BSEP) (liver, brain) (Daruich et al., 2019).

TUDCA has significant potential as a therapeutic. It is approved in Turkey and Italy for the treatment of cholesterol gallstones ("Tauroursodeoxycholic acid | C26H45NO6S - PubChem," n.d.) and UDCA (converted to TUDCA in vivo) was used safely as a hepatoprotective agent in a 10-year study for primary biliary cirrhosis/cholestatic liver disease (Poupon et al., 1999). TUDCA's mechanism of action for treating cholestasis is proposed to occur through its interaction with the membrane receptor α5β1 integrin, as previously mentioned. Activation of intrahepatocytic α5β1 integrin leads to activation of hepatoprotective signaling pathways via ERK1/2, focal adhesion kinase (FAK), and p38 (Gohlke et al., 2013). It is worth noting that this integrin or a homologue may also be expressed in yeast; antibody cross reactivity has been demonstrated between α5β1 integrin and alcohol dehydrogenase in *Candida albicans* and *Saccharomyces cerevisiae* (Klotz et al., 2001; Santoni et al., 1994).

In addition to its well-described effects on liver disease, TUDCA has also been used to treat diseases associated with aberrant UPR/ER stress signaling. In a study on pulmonary fibrosis, TUDCA was found to decrease UPR signaling by decreasing activation of ATF6 and eIF2α (Omura et al., 2013). Another study showed that TUDCA decreased UPR signaling through all three branches of the UPR during exposure to tunicamycin (Berger and Haller, 2011). A study on type 1 diabetes, however, found the opposite; TUDCA appeared to improve cell survival through an increase in ATF6 levels in pancreatic β cells (Engin et al., 2013). It is worth noting that diabetic pancreatic β cells had a decreased baseline level of UPR signaling (lowered expression of XBP1 and ATF6 compared to

control), suggesting that although these results differed from others in the literature, the net effect was a restoration of UPR signaling to "normal" levels in all cases. It has been previously suggested that TUDCA may also reduce apoptosis by decreasing calcium release from intracellular compartments (including the ER) during exposure to the ER stressor thapsigargin, thereby reducing calcium-dependent caspase 12 activation (Xie et al., 2002). Taken together, these data suggest that TUDCA may reduce cellular sensitivity to ER stress and reduce cell death, possibly via the UPR's apoptotic pathway. Given the connection between the UPR and other stress response systems (HSR, CWI), however, it is possible that TUDCA's mechanism of action may involve the modulation of a combination of ER stress, the UPR, and other related pathways.

1.12 Objectives and Hypotheses

Since proper protein folding, trafficking, and degradation are essential for protein function and therefore nearly every cellular (and organismal) activity, defects in these processes can lead to widespread consequences. Cellular quality control and stress response mechanisms which can resolve misfolded or unfolded protein accumulation are generally effective, but can be made less so by factors such as age, concurrent exposure to other stressors, and the length of time that the stress persists. To better understand this, we chose to utilize the budding yeast *Saccharomyces cerevisiae,* which has been used extensively to uncover the genetic mechanisms that control basic cellular processes, including survival, maintenance, and response to stressors. Therefore, using a yeast model, the work presented in this thesis focuses on the identification of genetic and environmental factors that decrease ER stress tolerance and lifespan, and drugs or small molecules that can increase stress tolerance. The central hypothesis of this thesis is that ***unlike replicative aging, chronological aging will require functional UPR for cellular tolerance and regulation of endoplasmic reticulum stress, and that this stress tolerance can be modulated with pharmacological interventions such as TUDCA.***

1.12.1 Chapter 2: A toolbox for rapid quantitative assessment of chronological lifespan and survival in *Saccharomyces cerevisiae*

Rationale: Understanding the factors which can alter yeast chronological aging phenotypes and lifespan could allow for a better understanding of the UPR and aging-related disease. However, existing methods for quantitative analysis of yeast lifespan could be improved upon in terms of efficiency, cost, and sample number. By developing a toolbox of quantitative, fluorescence-based techniques and analysis software to measure yeast chronological lifespan in a high-throughput but cost-effective manner, we will be able to study aging more efficiently and in greater detail.

Objective: Develop new fluorescence-based assays to quantitatively assess yeast chronological lifespan.

Hypothesis: The use of the new yeast viability assays will advance the field of research by offering faster, more accurate, less expensive, and higher throughput analysis of survival rates, and the generation of direct quantitative data.

1.12.2 Chapter 3: A functional unfolded protein response is required for chronological aging in *Saccharomyces cerevisiae*

Rationale: Many factors contribute to aging, including accumulation of toxic misfolded secretory proteins in the endoplasmic reticulum (ER stress), to which the cell responds through activation of ER stress signaling pathways such as the Unfolded Protein Response (UPR) via its effector protein, Ire1 (Back et al., 2005). Misfolded proteins and impaired UPR have been identified as factors in several age-related neurodegenerative diseases, such as Huntington's disease and Alzheimer's disease, making this field of study increasingly important as the population ages (Duennwald and Lindquist, 2008; Hoozemans et al., 2009; Scheper and Hoozemans, 2015). While the UPR has been shown to be non-essential for yeast replicative aging, its role in yeast chronological lifespan is not understood. Using a yeast model of aging and the viability methods developed in Chapter 2, we aimed to investigate the link between the UPR, ER stress, and CLS.

Objective: Define how ER stress and the UPR modulate chronological lifespan in a yeast model of aging, and understand how the unfolded protein response and ER-associated degradation impact ER homeostasis.

Hypothesis: Unlike what has been seen in replicative aging studies, dysregulation of endoplasmic reticulum stress responses during chronological aging will cause age-dependent cellular toxicity.

1.12.3 Chapter 4: Regulation of ER stress resistance by tauroursodeoxycholic acid in yeast

Rationale: Misfolded proteins and impaired UPR play a significant role in several age-related diseases, including diabetes and neurodegenerative diseases such as Parkinson's, Huntington's, and Alzheimer's diseases (Duennwald and Lindquist, 2008; Engin et al., 2013; Hoozemans et al., 2009; Ozcan et al., 2004). Tauroursodeoxycholic acid (TUDCA), a bear bile acid used for centuries as a traditional Chinese remedy, has been recently shown to improve symptoms and slow progression in these conditions. Despite promising results, TUDCA's mechanism of action is unknown. Given the connection between protein misfolding diseases and those improved by TUDCA, we propose that TUDCA may act by decreasing misfolded protein burden, lessening sensitivity to ER stress. Using a yeast model, we aimed to investigate the link between TUDCA, the UPR, and ER stress.

Objective: Decipher the mechanisms underlying the ability of TUDCA to alleviate ER stress.

Hypothesis: Treatment with TUDCA will decrease cellular sensitivity to ER stress by affecting cellular response to ER stress conditions, or by acting as a chemical chaperone to reduce misfolded protein burden.

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Chapter 2

2 A toolbox for rapid quantitative assessment of chronological lifespan and survival in *Saccharomyces cerevisiae*

Yeast chronological lifespan (CLS) assays offer the opportunity to study the impact of genetic or environmental factors on the aging process of eukaryotic cells. While effective, traditional measures of yeast CLS are often reliant on the ability of cells to divide or can be difficult to perform in a high throughput manner without specialized equipment. The work presented in this chapter sought to improve upon and adapt existing measures of yeast CLS, offering a toolbox of assays based on fluorescent viability dyes and an accompanying software program (ANALYSR) which allows for rapid data analysis and generation of survival percentages and curves[1].

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[1]A version of this chapter has been published:

Chadwick, S.R., Pananos, A.D., Di Gregorio, S.E., Park, A.E., Etedali-Zadeh, P., Duennwald, M.L., Lajoie, P., 2016. A Toolbox for Rapid Quantitative Assessment of Chronological Lifespan and Survival in *Saccharomyces cerevisiae*. Traffic 17, 689–703. doi:10.1111/tra.12391

2.1 Introduction

The budding yeast *Saccharomyces cerevisiae* has been used successfully as a model organism to study the basis of genetic regulation of cellular homeostasis (Kaeberlein et al., 2007). The availability of yeast deletion and overexpression collections provides scientists with an unmatched model system to study gene regulation and cellular signaling. Among the key cell features studied in yeast are the mechanisms regulating longevity (Burtner et al., 2011, 2009a; Jo et al., 2015; Kaeberlein et al., 2007, 2001; Longo et al., 2012; Mirisola et al., 2014; Murakami and Kaeberlein, 2009; Sutphin et al., 2012). Yeast is a powerful and well-established model to study aging, including basic aspects that are directly relevant to human aging. Yet how can aging studies in yeast help us understand what is happening in humans? Martin et al. proposed that highly conserved genes and mechanisms should be referred as "public mechanisms" of aging as opposed to "private mechanisms" which are restricted to distinct organisms (Martin et al., 1996). Public mechanisms include basic, highly-conserved pathways initially discovered in yeast, such as caloric restriction (Jiang et al., 2000; Kaeberlein et al., 2005) and the Ras/PKA pathway(Longo et al., 1999). Further, the role of sirtuins (Kennedy et al., 1995), which regulate aging in mammals, was first discovered in yeast.

Yeast allow the study of two distinct paradigms of aging: chronological and replicative lifespan (Longo et al., 2012, 1996; Piper, 2006) (**Figure 2.1**). Replicative lifespan is defined by the number of daughter cells a mother cell can produce before entering senescence (Mortimer and Johnston, 1959). Chronological lifespan (CLS) is defined as the amount of time non-dividing cells can survive in the absence of nutrients. Chronological lifespan experiments are typically performed by culturing cells for extensive periods of time at stationary phase. During the course of chronological aging, yeast cells undergo distinct growth phases: the mid-log phase (comprised of a mixture of dividing cells and quiescent cells), diauxic shift (metabolic shift transitioning into stationary phase), and stationary phase. Each of these growth phases is characterized by distinct metabolic activities and gene expression profiles (Goldberg et al., 2009), which

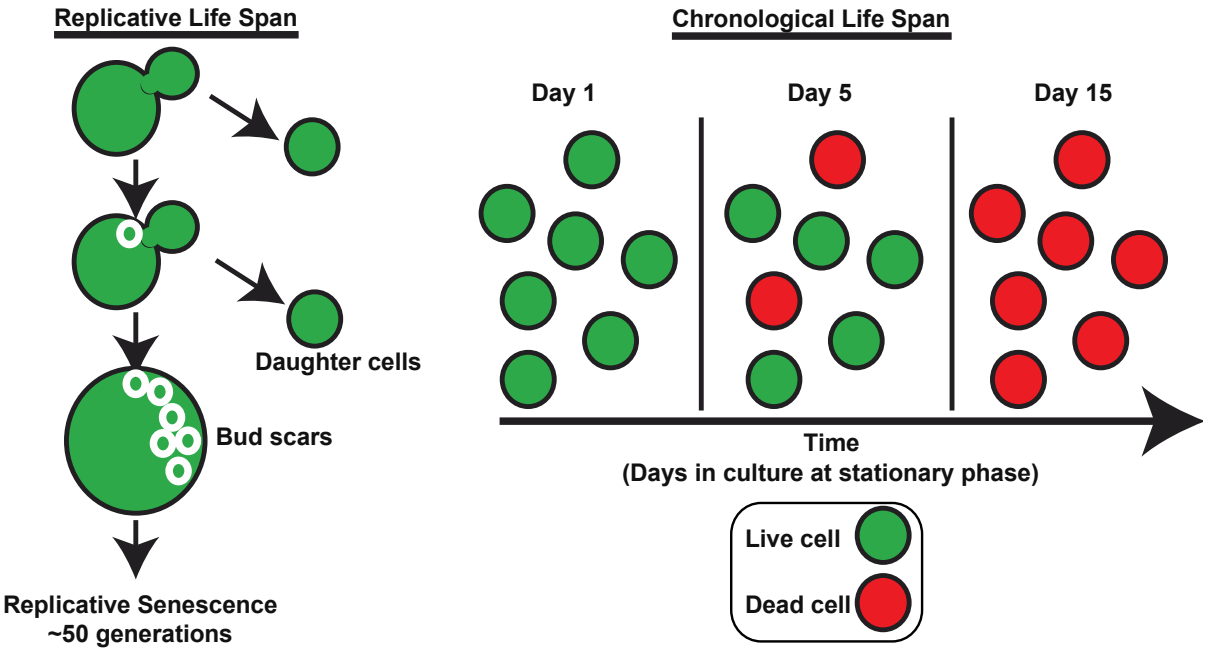

Figure 2.1: Models of yeast aging. Aging in yeast can be studied using two different paradigms: replicative or chronological lifespan. Replicative lifespan (RLS) is defined as the number of times a mother cell can produce daughter cells before entering replicative senescence. Chronological lifespan (CLS) is the amount of time a yeast cell can survive in a non-dividing state at stationary phase. CLS has been developed to model aging of post-mitotic cells in higher eukaryotes, such as neurons.

parallel central aspects of aging mammalian cells, such as increased respiratory activity, arrested cell cycle, and the accumulation of damaged proteins (Tissenbaum and Guarente, 2002). This model was developed to serve as a genetically and biochemically tractable model for aging of non-dividing cells in metazoans, such as neurons (MacLean et al., 2001).

CLS is traditionally assessed by aging cell cultures for several days in liquid media to allow cells to enter stationary phase. Every few days, an aliquot is removed from each culture, diluted, and plated on rich media-containing plates. The proportion of viable cells within the culture is determined based on the number a colonies growing on the plate (termed colony forming units, or CFUs) (Hu et al., 2013). While this method is well established, it requires a large quantity of agar plates, is time consuming, and is not suitable for high-content analysis. In order to circumvent these problems, scientists have established variations of this assay where the outgrowth of aging culture is performed in liquid media (Powers et al., 2006). The use of an incubator/shaker/plate reader system allows one to measure the optical density of the cultures over time and generate growth curves (Murakami et al., 2011). From these growth curves, one can use computer algorithms such as the yeast outgrowth data analysis (YODA) (Olsen et al., 2010) or GATHODE and CATHODE (Jung et al., 2015) programs to calculate the fraction of viable cells in the aging culture. This method, while less quantitative than the CFU methods, allows for high-content screening of yeast libraries, and requires fewer consumables and less time commitment. In addition, a competition survival-based assay has been developed wherein different strains are tagged with fluorescent proteins and cultured simultaneously to assess CLS using flow cytometry analysis of outgrown co-cultures (Garay et al., 2014). However, all these methods rely on the ability of the cells to re-enter the cell cycle and therefore only indirectly assess viability of aged cells.

It is possible to use a more direct method to measure cell death in aging culture by labeling cells with viability dyes (such as membrane-impermanent propidium iodide) that only stain dead cells. Dead cells can then be visualized using standard fluorescent microscopy. Again, the drawback of these methods is the significant amount of time it takes to acquire and quantify fluorescent images. Such fluorescently labeled cultures can

also be assessed using flow cytometry (Ocampo and Barrientos, 2011). While this has proven to be efficient, it does not accommodate high-content experiments unless the facility is equipped with a flow cytometer paired with a high content sampler capable of accommodating multi-well plates.

Here we describe methods to quantitatively monitor cell viability in aging cultures using fluorescent labeling of dead cells. We developed protocols optimized for quantification of cell viability in 96 well plates using a fluorescent cell counter, a simple UV trans-illuminator, or a fluorescent plate reader. We designed a simple computer program to enable rapid and efficient quantitation of fluorescent data that can be employed to assess a multitude of genetic or pharmacological perturbations. As a proof of principle, we used these methods to assess the effects of caloric restriction on CLS and found robust extension of lifespan, which is independent of the expression of three heat shock proteins (Hsp26, 42 and 104). Thus, our study provides researchers with a toolbox of reliable fluorescence-based assays for cost-efficient and large-scale analysis of CLS in yeast.

2.2 Results

2.2.1 Quantitative assessment of yeast chronological lifespan using a fluorescent cell counter

Yeast cell viability can be determined by labeling dead cells with fluorescent dyes such as propidium iodide (PI) (Krishan, 1975; Wallen et al., 1983). These fluorescent nucleic acid stains are known to be membrane-impermeant and therefore are only internalized by cells with compromised plasma membrane, a hallmark of terminal yeast cell death (Corliss and White, 1981). Upon incubation with PI, cells can be analyzed using either fluorescent microscopy or flow cytometry. In contrast, we employed a newly commercially available fluorescent cell counter (Countess II FL) to collect fluorescent images of PI-labeled yeast cultures. In this method, cells are labeled with PI and 10 µL of the culture is loaded onto a counting chamber. All images are collected using the same exposure time (**Figure 2.2A**). A negative control (unstained cells) and a positive control

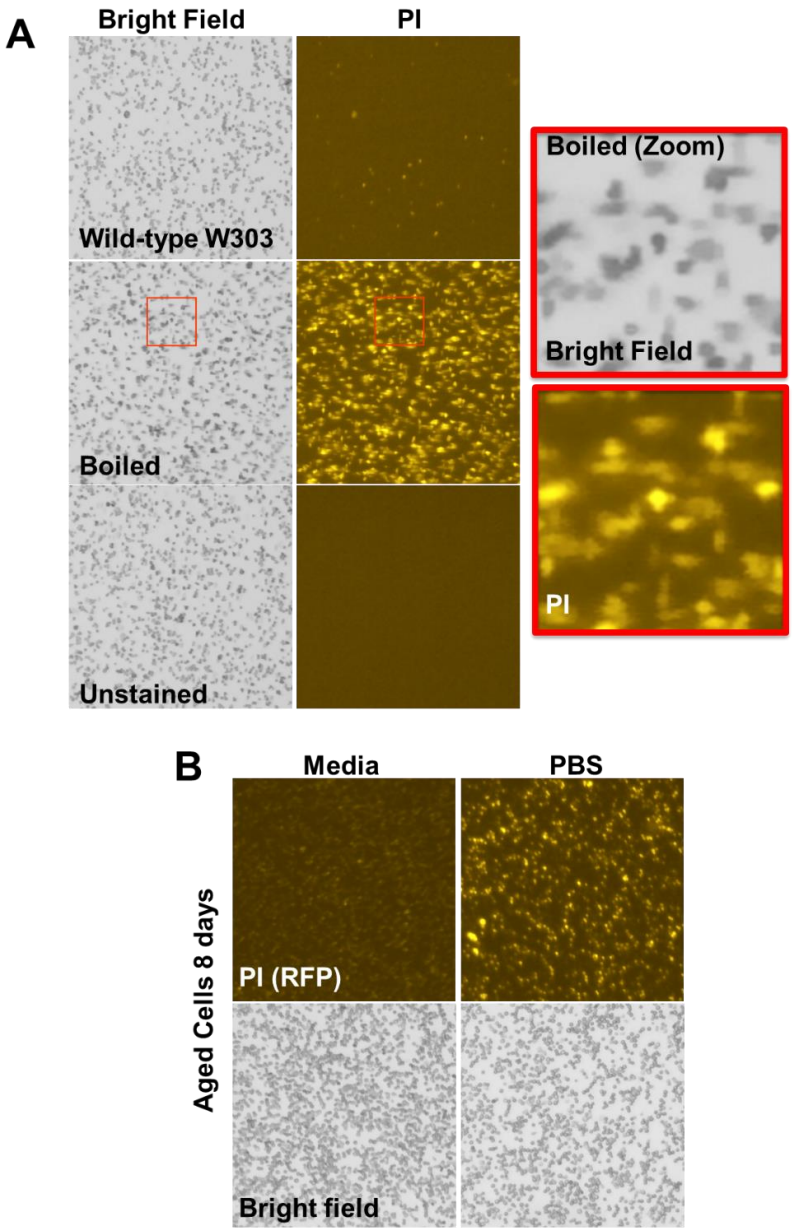

Figure 2.2: Propidium iodide staining and imaging. **A)** Cells aged from overnight cultures were stained with propidium iodide and imaged with the Countess II Cell Counter. Bright field images depict all cells in the sample, RFP images depict only fluorescently labelled cells. Boiled cells were used to depict 100% death; unstained cells represent 0% death. **B)** Cells aged for 8 days were stained with propidium iodide, either in original media or after being resuspended in PBS, and imaged with the Countess II Cell Counter.

(consisting of boiled cells) are included. Both bright field and fluorescent images are acquired. Interestingly, we found that PI staining in aging cultures is much more efficient when performed in PBS rather than in the standard yeast culture media (**Figure 2.2B**). We therefore suggest that samples be spun down and resuspended in PBS prior to staining. Although the counter can generate percent viability data, we obtained more consistent results by manually analyzing the raw images in ImageJ. Survival was calculated by measuring the area covered by PI-labeled cells in the red channel divided by the area covered by all cells in the bright field image. As proof of principal, this method was used to assess the effects of caloric restriction on yeast chronological aging. In yeast, caloric restriction can be achieved by reducing the concentration of glucose in the growth media and is known to extend lifespan (Kaeberlein et al., 2005, 2002; Murakami et al., 2008; Tsuchiya et al., 2006). Thus, yeast cells (W303a strain) were cultured in synthetic complete media for several days and viability was measured at different time points during the aging process with PI (**Figure 2.3A**). Cells cultured in media containing 0.1% glucose (as opposed to the standard 2%) were used to perform caloric restriction. We found that caloric restriction significantly increased lifespan, demonstrated by both percent survival and survival integral data (the area under the survival curve) (**Figure 2.3B and C**). Importantly, the maximal lifespan of W303a strain (~15 days) using this study was similar to previously published data using either regrowth assay or flow cytometry (Jung et al., 2015; Murakami and Kaeberlein, 2009; Ocampo and Barrientos, 2011). Previous studies have shown, however, that PI staining is not suitable for extreme caloric restriction experiments using water instead of growth media (Pereira and Saraiva, 2013). Thus, researchers should be cautious in the choice of methodology employed according to their type of study, and confirmation using other methods is suitable. While this simple method is suitable to quantify survival at stationary phase, it does not offer the ability to perform high throughput analysis. Therefore, we next sought to develop an assay that would allow detection of a high number of fluorescent samples simultaneously without the need of expensive automated instrumentation such as a flow cytometer.

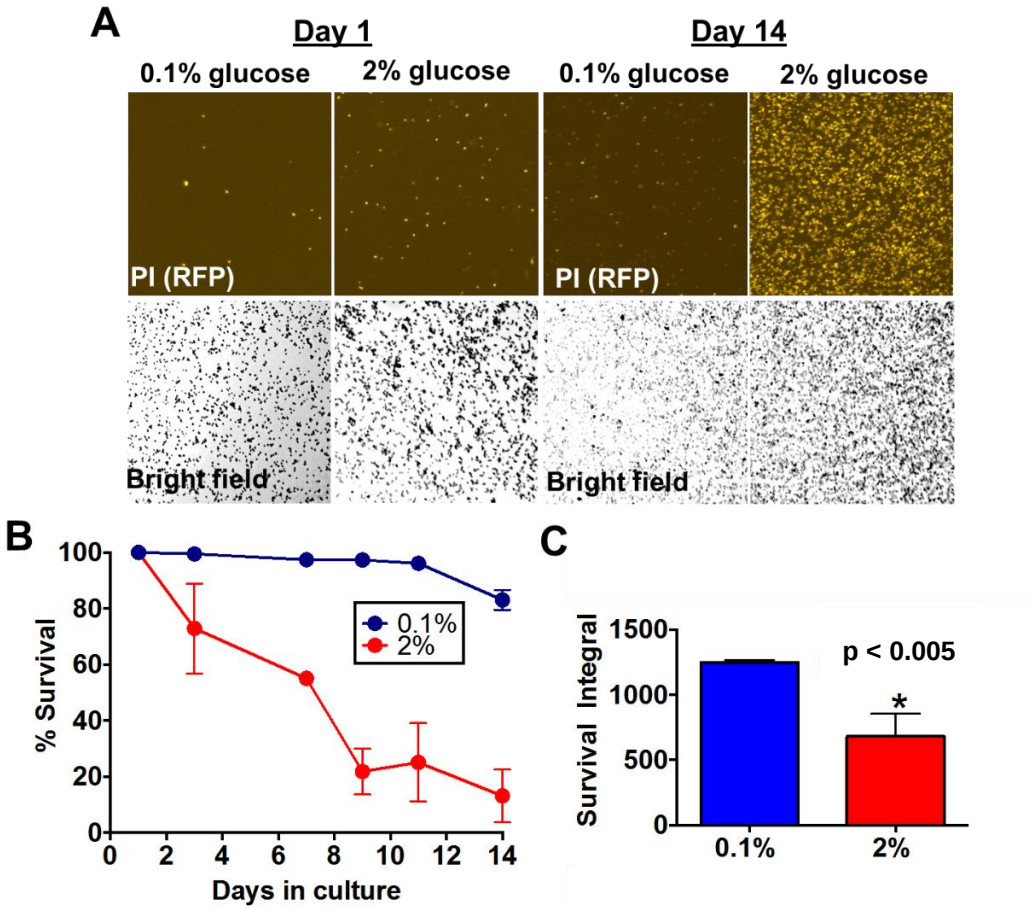

Figure 2.3: Effects of caloric restriction on CLS using the cell counter. Cells were aged in either standard synthetic media (2% glucose) or caloric restricted synthetic media (0.1% glucose) for the indicated time points before being stained with propidium iodide to measure percent death. Boiled cells were used as a positive control. Cells were imaged using the Countess II Cell Counter and analyzed with ImageJ. **A)** Side-by-side comparison of cell counter images obtained from days 1 and 14, from cells aged in standard (2% glucose) media and caloric restricted (0.1% glucose) media. Bright field images depict all cells in the sample, RFP images depict only stained (dead) cells. **B)** Using the ANALYSR computer program, normalized survival rates were generated based on data from cell counter images; data were normalized by absorbance and by positive and negative controls, with day 1 set at 100% survival. Survival integrals were also generated for mean normalized survival rates (± SD, n=6) **(C)** and compared using an unpaired t test.

2.2.2 Assessing CLS using fluorescence in multiwell plates

PI has an excitation peak at 350 nm, which makes it suitable for imaging using UV light. Thus, we tested if PI fluorescence could be detected in 96 well plates imaged with a UV transilluminator. Interestingly, we found that indeed, dead (boiled) cells stained with PI gave a significantly increased signal over unstained cells in a concentration dependent manner (**Figure 2.4A**). We quantified the mean fluorescent signal in each well using ImageJ software. Data were expressed in percent survival by normalizing to the signal of boiled cells (0% survival) and subtracting background signal from the unstained sample. In order to account for differences in cell population density among replicates, the same plate was also scanned using a plate reader to measure the absorbance at 595 nm (to measure cell density) and all samples were normalized to cell numbers. Several samples per condition were assessed in order to determine the variability within the experiment. Importantly, our assay was sensitive enough to detect as low as 10% cell death in a yeast culture (**Figure 2.4B**). This was determined by mixing increasing proportions of dead (boiled) cells stained with PI with live unstained cells prior to imaging. Next, we used this assay to measure survival at stationary phase. We subjected W303a cells to chronological aging. As expected, the intensity of PI staining increased significantly during chronological aging (**Figure 2.4C**), indicating that this method can be used to quantitatively assess CLS. Importantly, our data produced a maximal lifespan of ~15 days and reflected the known maximal lifespan of W303a cells (**Figure 2.4D**) (Jung et al., 2015; Murakami and Kaeberlein, 2009; Ocampo and Barrientos, 2011). Next, we used our multiwell assay to compare survival at stationary phase in standard (2% glucose) and caloric restricted (0.1% glucose) media, to recapitulate the lifespan extension describe before and reproduced using our cell counter assay. Indeed, cells grown in caloric restricted media showed very little increase in PI staining intensity during chronological aging compared to those grown in standard media (**Figure 2.5A**). Importantly, we found that multiwell plate assay yields similar survival results as the ones obtained with the cell counter (**Figure 2.5B and C**) or by using a semi-quantitative CLS spotting assay (**Figure 2.5D**), highlighting the accuracy of this approach.

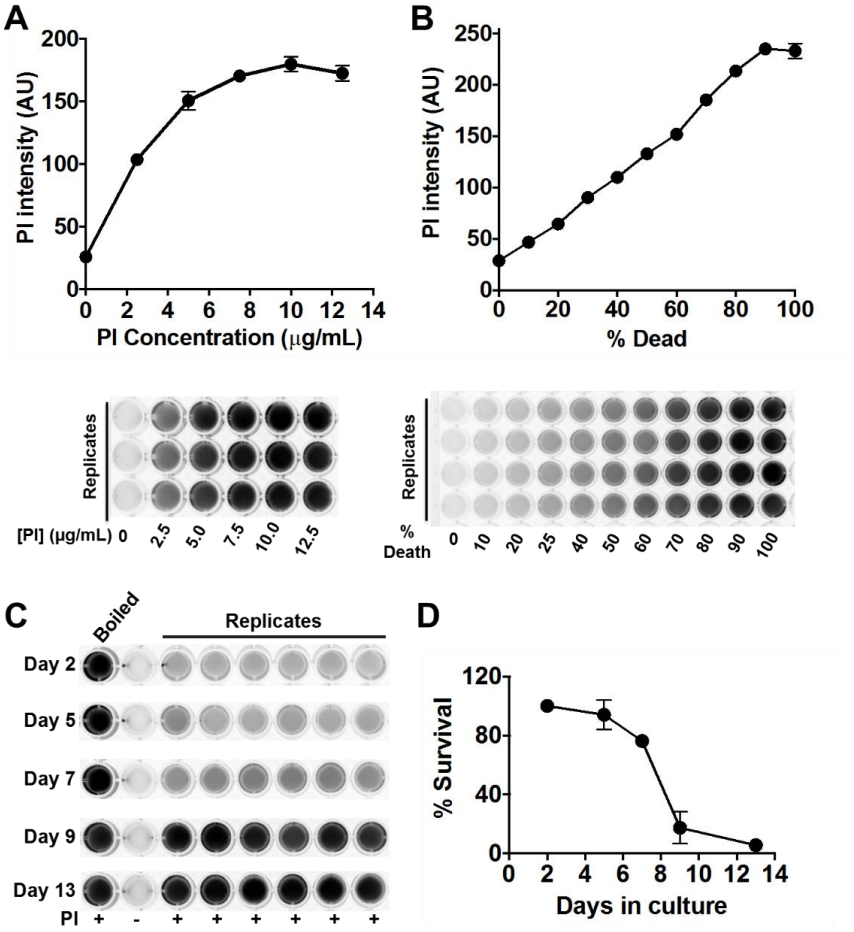

Figure 2.4: A fluorescence-based multiwell plate assay for CLS. Aged wild-type W303a cells were stained with propidium iodide and imaged with the UV transilluminator. **A)** Boiled cells were stained with different concentrations of PI and imaged to find the optimal PI concentration (n=3). **B)** Boiled, stained cells were mixed with different concentrations of unstained cells to produce different dilutions of PI-labeled dead cells. Four replicates of each dilution of stained cells were imaged. **C)** Wild-type cells were aged in synthetic media. At various time points, an aliquot was removed from each culture, stained, and imaged. One positive control, one negative control, and six replicates were analyzed at each time point. **(D)** Using the ANALYSR computer program, mean normalized survival rates (± SD, n=6) were generated based on fluorescent data; data were normalized by absorbance and by positive and negative controls, with day 1 set at 100% survival.

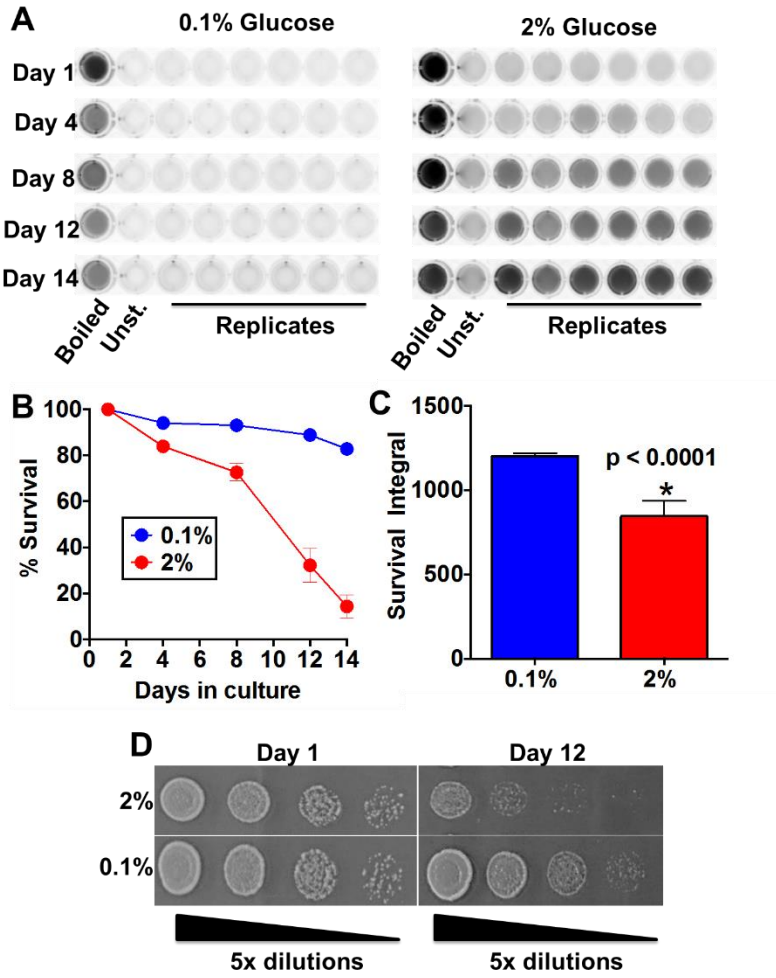

Figure 2.5: Quantification of lifespan extension by caloric restriction using the PI multiwell plate assay. Cells were aged in either standard synthetic media (2% glucose) or caloric restricted synthetic media (0.1% glucose) for the indicated time points before being stained with propidium iodide to measure percent death. **A)** 96 well plates were imaged with the transilluminator. One positive control, one negative control, and six replicates were analyzed at each time point. **(B)** Using the ANALYSR computer program, mean normalized survival rates (± SD, n=6) were generated based 96 well plate images; data were normalized by absorbance and by positive and negative controls, with day 1 set at 100% survival. **(C)** Survival integrals were calculated for each replicate and compared using an unpaired student's t-test (± SD, n=6). **(D)** Semi-quantitative CLS spot assay for wild-type W303a cells aged in either 2% or 0.1% glucose.

The fluorescence-based chronological lifespan assay described in this study allows rapid quantification of yeast survival during the aging process with the ability to assess viability of a large number of samples. This method also does not require any expensive equipment and can be performed using a simple UV gel box. In addition, it is significantly less labour-intensive when compared to the traditional CFU method. It also allows direct measurement of cell viability, as opposed to relying on the ability of cells to re-enter the cell cycle. Our method showed to be suitable for not only studying environmental or genetic perturbations that affect CLS, but also effects of toxic conditions. Indeed, we detected a concentration-dependent increase in toxicity of acetic acid (**Figure 2.6**), a chemical released by aging cells that is known to contribute to CLS (Burtner et al., 2009b). Wild-type yeast cells (W303a) were cultured in complete media for 4 days before being treated with acetic acid, which has previously been shown to be toxic to yeast (Burtner et al., 2009b). Cells were exposed to acetic acid ranging from 0-500 mM for 200 minutes before being stained with PI and imaged (**Figure 2.6A**). Quantitation showed that a distinct, linear decline in cell viability could be observed with increasing acetic acid concentration (**Figure 2.6B**). These data demonstrate that the methods described here are not limited to assessing CLS, but can be used to determine cell viability under a number of different conditions that induce cell death. It is important to note that our method using a UV transilluminator only allows the detection of one fluorescent dye/protein at the time. However, a fluorescent plate reader can be used to detect specific fluorescent signal using appropriate filter sets. We used the SYTOX Green viability dye in combination with a fluorescent plate reader and this method also highlighted the increased lifespan of caloric restricted cells (**Figure 2.7**). In addition, outgrowth assays, fluorescent microscopy, fluorescent cell counter or flow cytometry could be used. Therefore, our methods could be expanded for screening of yeast deletion collections or chemical libraries. Several studies have shown that yeast cells can be aged in multiwell plates, and our assay would be compatible with such protocols. However, generation of large-scale fluorescent data and subsequent analysis requires assistance of imaging software for rapid quantification. Thus, we generated freely available software that calculates survival rates in yeast cultures from fluorescent images.

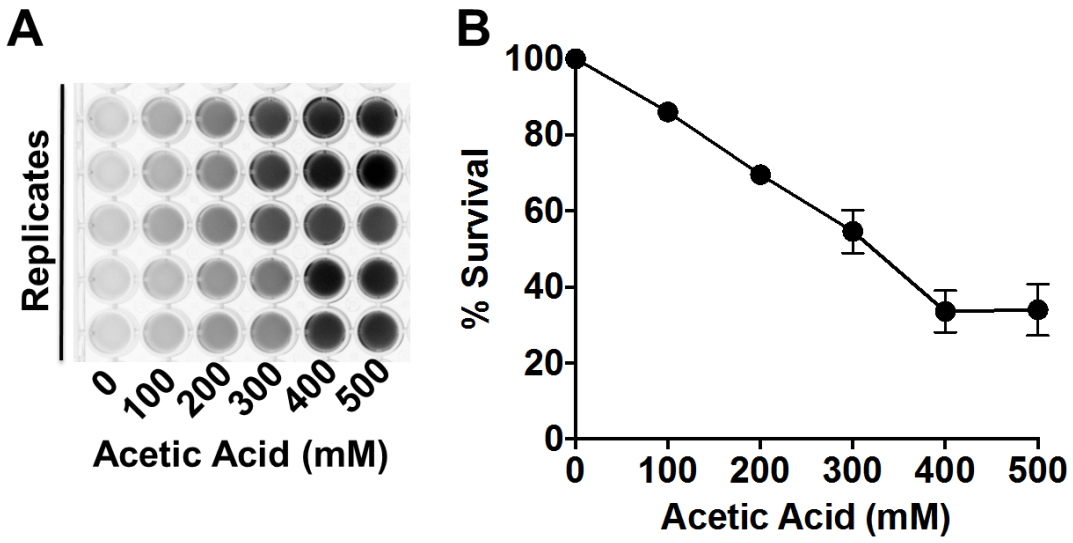

Figure 2.6: Acetic acid treatment induces cell death in yeast. WT cells were aged for 4 days before being treated with various concentrations of acetic acid for 200 minutes. **A)** Cells were stained with propidium iodide and imaged with the UV transilluminator. Five replicates were analyzed for each concentration. **B)** Mean normalized survival rates (± SD, n=5) based on data from UV transilluminator images using ANALYSR; data were normalized with absorbance and positive and negative controls, with untreated cells values set at 100% survival.

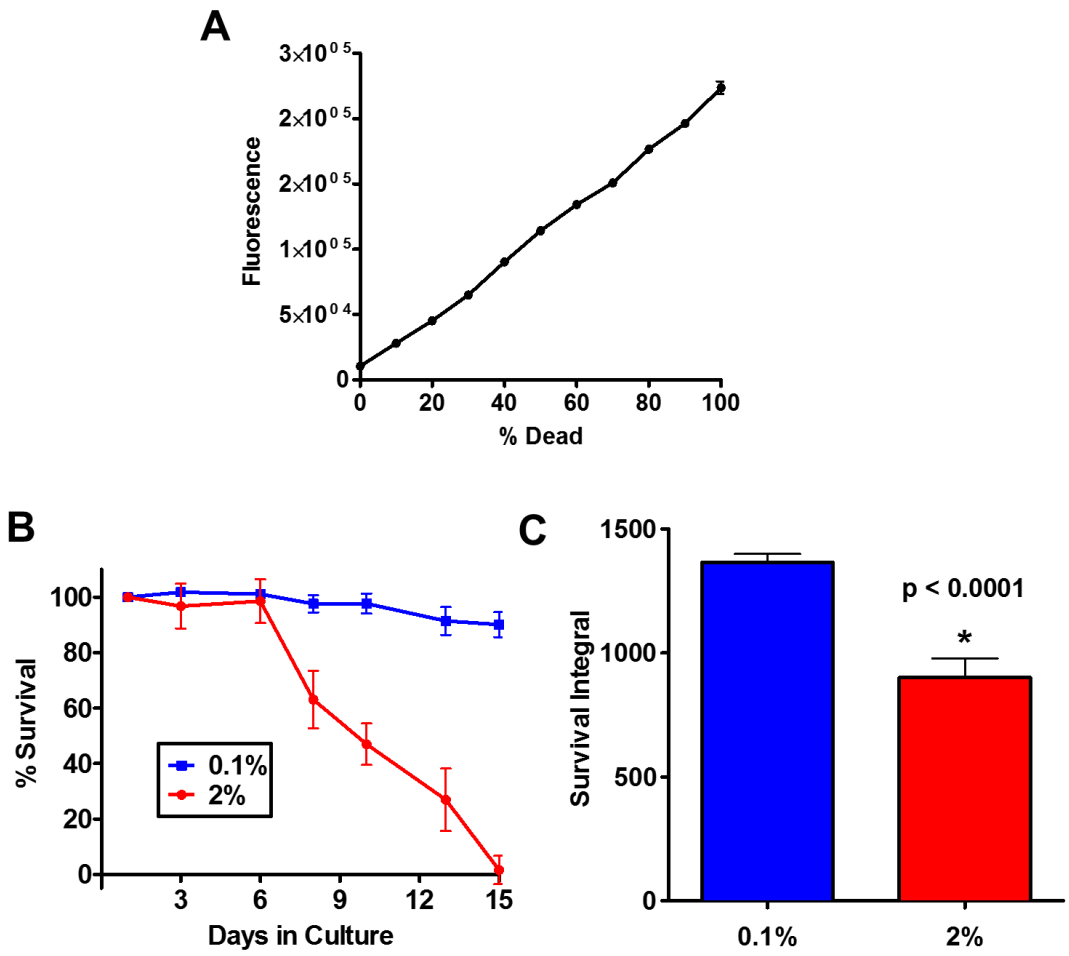

Figure 2.7: Quantification of lifespan extension by caloric restriction using a fluorescent plate reader. A) Boiled, stained cells were mixed with different concentrations of unstained cells to produce different dilutions of SYTOX Green-labeled dead cells. 4 replicates of each dilution of stained cells were imaged using a fluorescent plate reader. **(B)** Cells were aged in either standard synthetic media (2% glucose) or caloric restricted synthetic media (0.1% glucose) for the indicated time points before being stained with SYTOX Green to measure percent death. 96 well plates were imaged a fluorescent plate reader. Using the ANALYSR computer program, mean normalized survival rates (± SD, n=10) were generated based 96 well plate images; data were normalized by absorbance and by positive and negative controls, with day 1 set at 100% survival. **(C)** Survival integrals were calculated for each replicate and compared using an unpaired student's t-test (± SD, n=10).

2.2.3 Computer program for rapid quantification of CLS using fluorescent data

The first step of data analysis is to determine the fluorescent intensity within the various wells of a plate. This can be done using any available imaging software, such as ImageJ. The well to be analyzed is selected and the mean fluorescent intensity from that well is recorded. These data can be saved in a Microsoft Excel format and used to calculate survival rates. In order to perform such analysis, we designed a new open-source software named **ANALYSR** (**An**alytical **Al**gorithm for **Y**east **S**urvival **R**ates). This program was designed specifically for the purpose of analyzing data from fluorescence-based viability assays. The software runs in Python and accepts .xlsx files as input for the fluorescent data and optical density/absorbance (if required) for these assays over a number of days. Using these data, ANALYSR calculates the percent survival in each replicate of a given condition relative to the positive and negative controls. Then, the data are normalized to the day 1 of the CLS experiment (set at 100% survival). Users also have the option of normalizing each sample to its optical density/absorbance data. ANALYSR produces two Excel files: one with the raw data and one with the normalized percent survival data (**Figure 2.8A**). It also produces two graphs with standard error bars, one showing the trend in percent survival over time in each replicate separately, and the other showing this trend as the average of all replicates (**Figure 2.8B and C**). If a sample has a higher degree of fluorescence than the positive (boiled) sample, the percent survival is set at 0%; no negative percentages are calculated. The software also allows users to measure the area under the curve (survival integral) for each replicate using the trapezoid rule (wherein area is calculated by dividing the shape into trapezoids and calculating individual areas), allowing statistical comparison of survival rates between different conditions. The ANALYSR software was written in the Python programming language and the source code and instructions are freely available (**Appendix A**). The Python package can be run using Enthought Canopy, a freely available Python analysis environment.

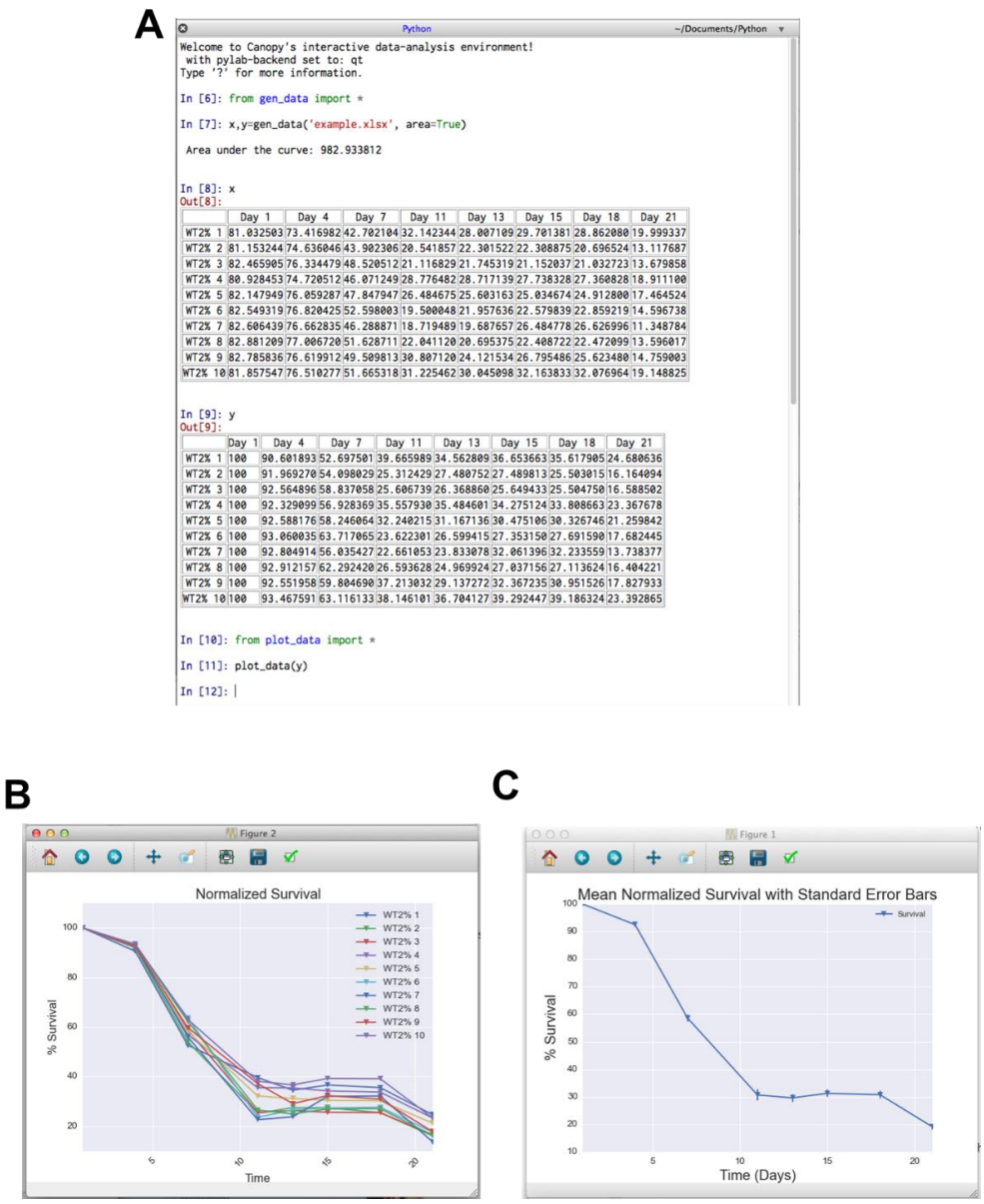

Figure 2.8: ANALYSR – Analytical Algorithm for Yeast Survival Rates A) A screenshot depicting the user interface and output from the python-based program designed for this research. Output includes percent survival data normalized only to positive and negative controls, and the same data with day 1 set to 100% survival. **B)** One chart produced by the program, showing the normalized survival of each replicate in one condition. **C)** Mean normalized survival chart produced by the program, including standard error bars.

2.2.4 Application: Hsp24, 42 and 104 are dispensable for lifespan extension by CR

To provide evidence that the methods presented are effective, we performed further experiments and assessed the impact of genetic mutations on CLS. The deletion of members of the Hsp31 minifamily has been previously shown to severely reduce CLS (Miller-Fleming et al., 2014). Whether other Hsp proteins are required for CLS is unclear. Thus, we used yeast cells carrying deletions of three heat shock proteins required for heat shock tolerance/protein disaggregation, but with an undefined role in CLS (*hsp104*Δ*, hsp42*Δ*, hsp26*Δ) (Cashikar et al., 2005). First, we tested the sensitivity of these cells to heat shock using both qualitative methods (spot assay) and our newly developed methods. Wild-type and triple Hsp deletion mutant cells were either left untreated or exposed to a tolerance-inducing heat shock at 37°C for 30 minutes followed by either 10- or 20-minute incubation at a lethal temperature (50°C) (Cashikar et al., 2005). While heat shock did impair growth of the triple deletion mutant during the spot assay (**Figure 2.9A**), we surprisingly found that it did not actually result in PI uptake by the cells, indicating that the plasma membrane was not compromised. PI staining revealed that, upon heat shock, the deletion mutant experienced modest cell death similar to what is observed in wild-type cells (**Figure 2.9B**). These data suggested that these heat shock proteins are not necessary for cell survival during heat shock, and are perhaps only required for later re-entering the cell cycle after heat shock. However, we did not observe PI uptake even 18 hours following heat shock (**Figure 2.9C**) suggesting that these cells may die from mechanisms that do not involved a compromised plasma membrane. At the same time, heat shock at higher temperature resulted in PI-positive cells even in wild-type, indicating that 50°C may represent a milder stress that can induce cell death via a different mechanism. Evidence in the literature shows that yeast cells may undergo apoptosis or apoptosis-like cell death, which is not necessarily associated with increased plasma membrane permeability (Aerts et al., 2009; Herker et al., 2004; Madeo et al., 1999). Indeed, we observed increased DNA fragmentation in the triple deletion mutant compared to wild-type following heat shock indicating that these cells may be undergoing apoptosis (**Figure 2.9D**). It is possible that heat shock induces apoptotic-like cell death, which can result in secondary necrosis (Carmona-Gutierrez et al.,

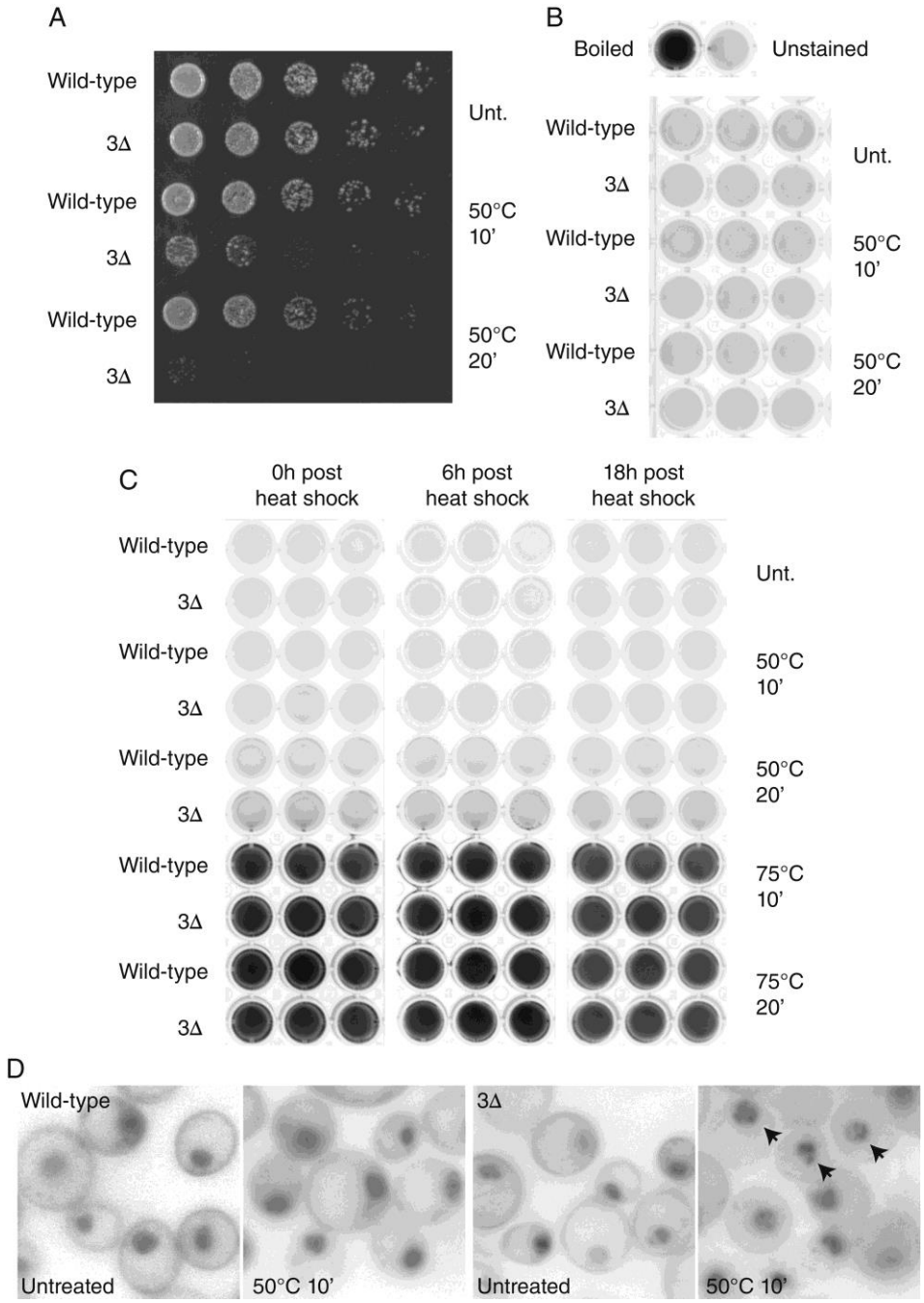

Figure 2.9: Comparison of regrowth assay vs. direct viability staining. **A)** Wild-type and *hsp104*Δ, *hsp42*Δ, *hsp26*Δ (3Δ) cells were left either untreated, or treated at tolerance-inducing temperature (37°C) for 30 min, followed by lethal heat shock at 50°C for either 10 or 20 min. Cells were then spotted on YPD plates. **B)** Wild-type and *hsp104*Δ, *hsp42*Δ, *hsp26*Δ (3Δ) subjected to 50°C heat shock for 10 or 20 min were stained with propidium iodide (triplicates) and imaged with the UV transilluminator. **C)**

Wild-type and *hsp104*Δ, *hsp42*Δ, *hsp26*Δ (3Δ) subjected to 50°C or 72°C heat shock for 10 or 20 min were stained with propidium iodide (triplicates) and imaged with the UV transilluminator. **D)** Wild-type and *hsp104*Δ, *hsp42*Δ, *hsp26*Δ (3Δ) cells were left either untreated, or treated at tolerance-inducing temperature (37°C) for 30 min, followed by lethal heat shock at 50°C for 10 min. Cells were then labeled with DAPI and imaged using a wide-field fluorescent microscope. Inverted black and white images are shown for clarity. 3Δ cells show increased DNA fragmentation (arrowheads) upon heat shock indicating that cells undergo apoptosis-like death.

2010) once the plasma membrane is compromised and may explain the PI staining observed at higher temperatures (**Figure 2.9C**). Thus, while the PI viability assay can provide additional information that cannot be obtained using spotting assays, it should also be used with care, as it might not always allow efficient detection of non-viable cells that do not have compromised plasma membrane.

Next, we assessed survival of the triple mutant at stationary phase. Interestingly, we found that deletions of these specific Hsp proteins did not significantly impair CLS (**Figure 2.10A**). Importantly, incubation in low glucose-containing media was still able to increase lifespan in the triple mutant using our assay and was confirmed using a semi-quantitative CLS (**Figure 2.10A-D**). These results indicate that, while these three Hsp proteins are known to be upregulated during stationary phase (Gasch et al., 2000), they are not required for extension of lifespan by caloric restriction. We then concluded that their function must be required for tolerance of others type of stresses (such as heat shock) as previously described (Sanchez et al., 1992) and highlighted in **Figure 2.9**. Our results also indicate that the requirement for Hsp104 is different between CLS and replicative lifespan, since the protein is highly involved in preventing transfer of misfolded protein aggregates from mother to daughter cells (Andersson et al., 2013; Hill et al., 2014; Saarikangas and Barral, 2015).

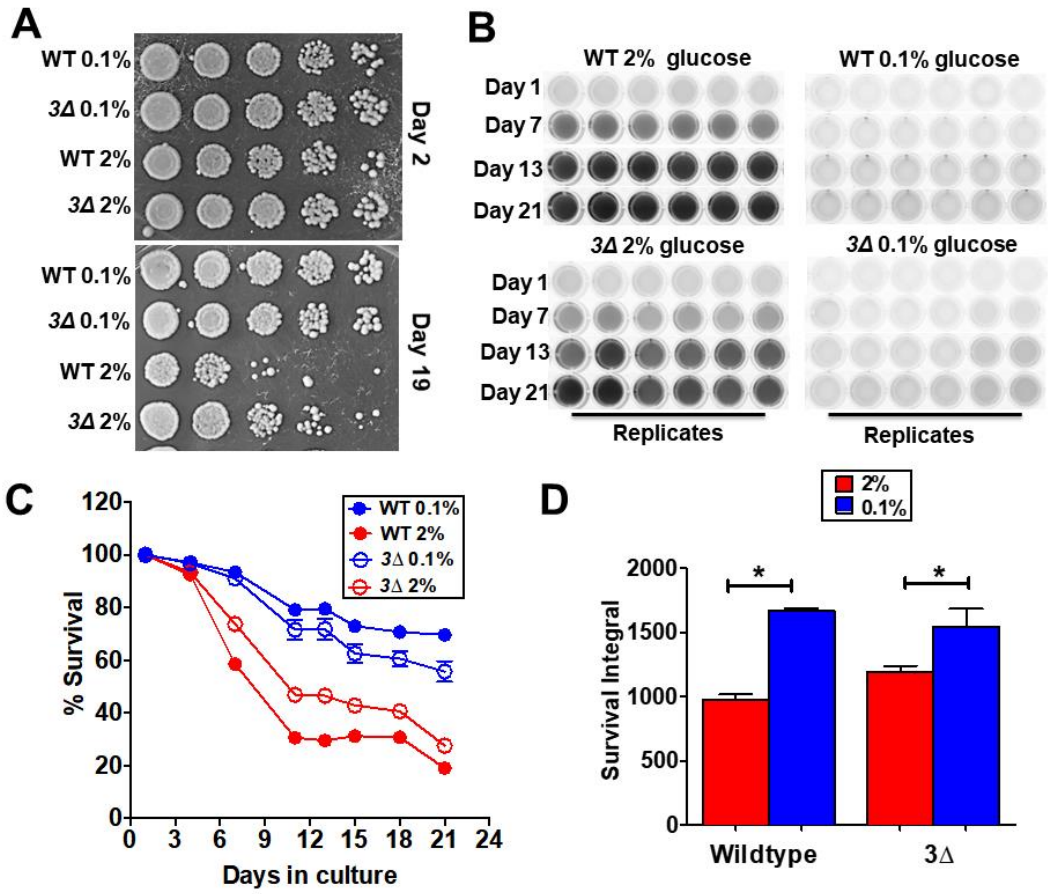

Figure 2.10 Hsp26, 42 and 104 are dispensable for lifespan extension by caloric restriction. **A)** Wild-type and *hsp104*Δ, *hsp42*Δ, *hsp26*Δ (3Δ) cells were aged for 21 days in either standard (2% glucose) or caloric restricted (0.1% glucose) synthetic media and spotted on YPD plates. **B)** Chronologically aged wild-type and *hsp104*Δ, *hsp42*Δ, *hsp26*Δ (3Δ) cells stained with PI in 96 well plates and imaged using the UV transilluminator. One positive control, one negative control, and ten replicates were analyzed at each time point. **C)** Normalized survival rates (± SD, n=10) were generated based on data from UV transilluminator images; data were normalized by absorbance and by positive and negative controls, with day 1 set at 100% survival. **D)** Survival integrals were generated for each condition and compared to cells in standard media (2% glucose) using 1-way ANOVA and Tukey's test ($P < 0.05$) (± SD, n=10).

2.3 Discussion

The methods described here have proven to be an effective way to assess chronological lifespan in yeast. Our new methods successfully confirmed the effects of caloric restriction on CLS determined by other methods. Using a 96-well plates and readily available technology (such as UV transilluminators), we were able to acquire large amounts of quantitative data relatively quickly, using far fewer resources than the traditional CFU method. This proved to be a significant advantage over traditional CFU assays, which require a high number of agar plates and a large time commitment. Another feature of our assay is the direct quantitation of cell viability, which offers a complementary measure of viability to the regrowth assays. Thus, we have provided researchers with a new toolbox of reliable fluorescence-based assays that allow rapid, cost-efficient and large-scale analysis of CLS in yeast.

These methods, while effective, also have limitations. It is not always possible, for example, to quantify cell viability of strains expressing fluorescent proteins (i.e. GFP), as the UV transilluminator cannot excite at specific wavelengths. In such cases, a fluorescent plate reader must be used to differentiate the fluorescent signals. The use of multiple assays in measuring CLS and cell viability is important, as results may vary between and within methods. It is therefore important to first assess the sensitivity of the plate reader or transilluminator and find the ideal concentration of nucleic acid dye before performing viability assays. Since these methods (and the accompanying ANALYSR software) are designed to measure viability during CLS experiments, they could be easily repurposed to suit other needs. They could also be utilized for measuring viability in other conditions, such as treatment with drugs or heat shock as we demonstrated.

In summary, our methods provide the opportunity for researchers to acquire high quality, high-throughput viability data. The availability of different yeast mutant collections (non-essential gene deletions, temperature sensitive alleles, overexpression libraries) offers the possibility to assess the roles of specific genes in the aging process on a large scale. Our assays could allow for large amounts of reliable quantitative data to be produced and offer a complementary approach to study performed using alternative CLS methods.

Finally, the use of the ANALYSR software is not limited to yeast and can be used to quantify any kind of viability data independently of the organism employed.

2.4 Materials and Methods

2.4.1 Cell culture and Chronological lifespan assay

Strains used in this study are listed in **Table 2.1**. For every experiment, W303a *Saccharomyces cerevisae* cells or derivatives were thawed from frozen stocks and grown on YPD for two days at 30ºC before being transferred to liquid cultures. 5 ml of liquid cultures were grown to saturation overnight before being separated into replicates (100 µL of overnight cultures in 5 mL of liquid media) for CLS experiments. All experiments were carried out using synthetic complete media (SC). Replicates were incubated in polystyrene snap cap tubes at 30ºC in a rotating drum over the course of the aging process.

Table 2.1: Strains used in chapter 2

Strains	Genotype	Description	Reference
W303a	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	Wild-type	(Thomas and Rothstein, 1989)
AGC15	*MATα, leu2-3,112 trp1-1 ura3-1 ade2-1 his3-11,15 lys2D can1-100 hsp26::LEU2 hsp104::KanMX, hsp42::HygB*	*hsp26*Δ/*hsp42*Δ/ *hsp104*Δ	(Cashikar et al., 2005)

2.4.2 Cell viability staining

200 µL of each cell culture was added to 1.5 mL tubes, plus two extra samples to serve as positive and negative controls. The positive control was boiled for 10 minutes at 100ºC. All samples were pelleted for 2.5 minutes. All samples except the negative control were resuspended in 200 µL of phosphate-buffered saline (PBS) containing a 1/200 dilution of a propidium iodide stock solution (1 mg/ml in H_2O). The negative control was resuspended in 200 µL of PBS alone. The samples were incubated at room temperature

for 10 minutes. Imaging was then performed using either the Countess II FL cell counter (Life Technologies), a Gel Doc system (BioRad) or a Victor2 plate reader (Perkin Elmer). The optical density (OD_{600}) of each sample was determined with either plate reader, or by measuring the absorbance of diluted samples in cuvettes. Survival rates and integrals were calculated using the ANALYSR program described in this manuscript (see Appendix A for extended instructions).

2.4.3 Semi-quantitative CLS assay (Spot Assay)

Throughout the chronological aging process, an aliquot of the aging cultures was removed and 5x serial dilutions of OD_{600} 0.2 were spotted onto YPD agar plates. Plates were then incubated at 30°C for 2-3 days and imaged using the BioRad Gel Doc system.

2.4.4 Fluorescent cell counter

For imaging, 10 µL of each stained sample was loaded onto a reusable hemacytometer and inserted into a Countess II FL Automated Cell Counter. Bright field and RFP/GFP (depending on stain used) images were obtained and manually quantified, as the cell counter's ability to distinguish small budding yeast cells was determined to be unreliable with the current software. To quantify, the positive control images for a given condition were opened first in ImageJ (http://imagej.nih.gov/ij/). The threshold was set for each image type, bright field and RFP, ensuring all cells were covered, and "Analyze Particles" was selected. The percent of the total area covered by the particles (cells) in the sample was recorded. From there, all other samples within that condition were analyzed using the same method, ensuring the threshold set matched that of the positive control. The resulting data were then analyzed using the ANALYSR program.

2.4.5 Imaging with UV transilluminator and fluorescent plate reader

For imaging, 200 µL of each propidium iodide-stained sample was loaded into a well in a 96-well plate. The plate was loaded into a BioRad Gel Doc and imaged at 300 nm. Exposure time differed between plates, but the use of a positive control (boiled sample) allowed for normalization. The images were analyzed in ImageJ by selecting the sample within each well and measuring the mean grey value for each one. The data were then

analyzed via ANALYSR. Alternatively, cells were stained with SYTOX Green (Life Technologies) and plates were imaged with a fluorescent plate reader (Victor2, Perkin Elmer) equipped with a combination of 485 nm excitation filter and 535 nm emission filter for SYTOX Green imaging.

2.4.6 Fluorescent microscopy

Upon heat shock, cells were stained with 2.5 µg/ml DAPI for 30 minutes in growth media, then washed and resuspended in PBS and imaged in LABTEK (Nunc inc.) imaging chambers using a Zeiss Axiovert A1 wide-field fluorescent microscope equipped with a 365 nm excitation/420 emission long pass filter for DAPI and a 63X 1.4 numerical aperture oil objective and a AxioCam ICm1 R1 CCD camera (Carl Zeiss inc.). Images were analyzed using ImageJ.

2.5 Acknowledgements

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Chapter 3

3 A functional unfolded protein response is required for chronological aging in *Saccharomyces cerevisiae*

The UPR plays a wide variety of roles in health and disease and has been demonstrated to change over the course of aging in mammalian and invertebrate models. It has been previously shown that the UPR does not regulate yeast replicative lifespan; the role of the UPR in yeast chronological lifespan, however, is not well understood. Using the methods developed in Chapter 2 which allow for rapid assessment of yeast CLS, this chapter aimed to study chronological aging in more depth, working to determine whether functional UPR was required for chronological aging, and to identify which specific components of the UPR signaling pathway were involved in regulating CLS[2].

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[2] A version of this chapter has been published:

Chadwick, S.R., Fazio, E.N., Etedali-Zadeh, P., Genereaux, J., Duennwald, M.L., Lajoie, P., 2019. A functional unfolded protein response is required for chronological aging in *Saccharomyces cerevisiae*. Curr Genet. doi:10.1007/s00294-019-01019-0

3.1 Introduction

Nearly one third of the eukaryotic cell proteome consists of secretory proteins that enter the endoplasmic reticulum (ER) upon synthesis. In order to ensure proper folding and protein quality control (PQC) of the nascent polypeptides, cells have evolved a complex network of proteins that monitor the ER misfolded protein burden (Walter and Ron, 2011). High levels of misfolded proteins can overwhelm the capacity of the PQC machinery, thus activating stress signaling pathways to adapt the ER folding environment. Among these pathways is the unfolded protein response (UPR), which is conserved from yeast to mammals (Ron and Walter, 2007; Walter and Ron, 2011). In yeast, the ER stress sensor, Ire1 (Inositol requiring enzyme 1), detects misfolded protein accumulation through its luminal domain (Gardner and Walter, 2011; Kimata et al., 2007). This results in its dimerization, trans-autophosphorylation (Aragón et al., 2009), and activation of its RNAse domain (Cox and Walter, 1996). The latter allows the splicing of the *HAC1* mRNA, which is translated into a functional transcription factor (Cox and Walter, 1996) that can upregulate nearly 400 genes covering a broad spectrum of functions (Travers et al., 2000) associated with ER QC, such as ER chaperones to assist protein folding (Mori et al., 1992), components of the ER associated-degradation (ERAD) machinery to eliminate misfolded peptides (Yoshida et al., 2003), and lipid synthesis to increase ER membrane proliferation (Schuck et al., 2009). Of note, the efficiency of this signaling, as well as the efficiency of the subsequent responses to ER stress, decreases during aging (Ben-Zvi et al., 2009; Brown and Naidoo, 2012; Chadwick and Lajoie, 2019).

Aging is an important modifier of the PQC network, meaning that aging cells have reduced capacity to properly carry out protein transcription, translation, folding, transport, and degradation (Brown and Naidoo, 2012; Chadwick and Lajoie, 2019; Naidoo, 2009). Aging cells have also been shown to have decreased levels of many ER QC proteins, including protein remodeling factors and molecular chaperones (such as Kar2/BiP), which normally ensure proper protein folding or target misfolded proteins for degradation (Hussain and Ramaiah, 2007; Paz Gavilán et al., 2006). Moreover, the remaining chaperone proteins present in the aging ER appear to be functionally impaired,

possibly due to progressive protein oxidation over time (Ma and Hendershot, 2004; Naidoo et al., 2008; Nuss et al., 2008; van der Vlies et al., 2003). Proteotoxic stress and aging can also affect the redox status of not only the ER, but globally across cellular compartments (Kirstein et al., 2015). These factors collectively lead to an impaired ability of aged cells to resolve and adapt to ER stress.

Inherited mutations, environmental stress, and reduced capacity of cells to cope with misfolded proteins are major factors that determine cellular and thus organismal ability to mitigate the damaging effects of the aging process (Brehme and Voisine, 2016; Höhn et al., 2017). For instance, studies in *C. elegans* support a role for a secreted ER stress signal in the control of stress resistance and lifespan expansion (Taylor and Dillin, 2013). Increased lifespan by the UPR effector XBP1 relies on the FOXO family transcription factor DAF-16, which is a major regulator of genes involved in metabolism and longevity in worms (Douglas et al., 2015; Henis-Korenblit et al., 2010). Similar studies in flies showed that UPR signaling is a major regulator of lifespan (Luis et al., 2016; Wang et al., 2015, 2014). Furthermore, the role of the UPR in aging is highlighted by the significant age-dependent toxicity observed in several misfolded protein-associated diseases, such as Huntington's and Alzheimer's disease (Gavilán et al., 2009; Williams and Paulson, 2008; Yang et al., 2014). Accumulation of misfolded proteins and UPR activation have been linked to aging (Brown and Naidoo, 2012; Salminen and Kaarniranta, 2010), yet it remains unclear how specific components of the PQC network contribute to a healthy lifespan under both normal and pathological conditions.

Yeast is well established as a model to study the genetic mechanisms of the aging process. Two different aging paradigms have been established in yeast: chronological and replicative aging (Denoth Lippuner et al., 2014; Kaeberlein et al., 2007). Replicative lifespan (RLS) is defined by the number of times a mother cell buds and generates daughter cells before entering replicative senescence (Jo et al., 2015; Steffen et al., 2009). Chronological aging, or chronological lifespan (CLS), is defined as the amount of time cells can survive in the non-dividing state at stationary phase (Chadwick et al., 2016; Herker et al., 2004; Laun et al., 2006; Murakami and Kaeberlein, 2009). Chronological aging thus serves as a tractable model for aging post-mitotic cells, such as neurons. Both

aging paradigms have led to the discoveries of specific pathways required for lifespan extension in metazoan organisms, such as TOR signaling and the role of sirtuins (Guarente and Kenyon, 2000; Jiang et al., 2000; Kennedy et al., 2005; Tsuchiya et al., 2006). Caloric restriction, for example, a well-established method to extend lifespan in mammalian cells and whole-animal systems, was first discovered in yeast (Colman et al., 2009; Jiang et al., 2000). Interestingly, yeast cells lacking a functional UPR (*ire1*Δ or *hac1*Δ) do not display changes in replicative lifespan (Labunskyy et al., 2014). However, the role of the UPR in chronological aging remains unclear. We therefore employed the yeast chronological aging model to investigate how activation of the UPR and other PQC pathways affect the aging process.

3.2 Results

3.2.1 Deletion of *IRE1* shortens chronological lifespan

Defects in cellular stress response pathways have been shown to modulate lifespan in yeast and other organisms (Kapahi et al., 2004; Postnikoff et al., 2017). We first investigated the importance of the UPR and growth conditions on yeast chronological lifespan (CLS). Cells that lack a functional UPR (e.g. *ire1*Δ) are known to display increased sensitivity to ER stressors, such as treatment with the N-glycosylation inhibitor, tunicamycin, and are inositol auxotrophs (**Figure 3.1A and B**) (Cox et al., 1993; Mori et al., 1993; Nikawa and Yamashita, 1992). CLS was measured using propidium iodide staining to assess cell death, and survival curves and survival integrals (i.e. the area under the curve for each survival curve (Burtner et al., 2009a; Chadwick et al., 2016)) to assess general fitness were generated for each condition. It should be noted that genetic background and plasmid backbones used can alter the absolute lifespan of different strains; internal controls are included within each experiment to account for this. We found that *ire1*Δ cells remain viable during logarithmic growth until they reach stationary phase when they display significantly reduced lifespan, as measured by both cell viability and spot assay (**Figure 3.1C and D**). To account for the possibility that aging defects seen in *ire1*Δ cells are attributable to a difference in viability during the early phases of the aging process (late log phase and post-diauxic shift), these data were also analyzed using day 3 of growth normalization set to 100% (**Supplemental Figure 3.1A and B**).

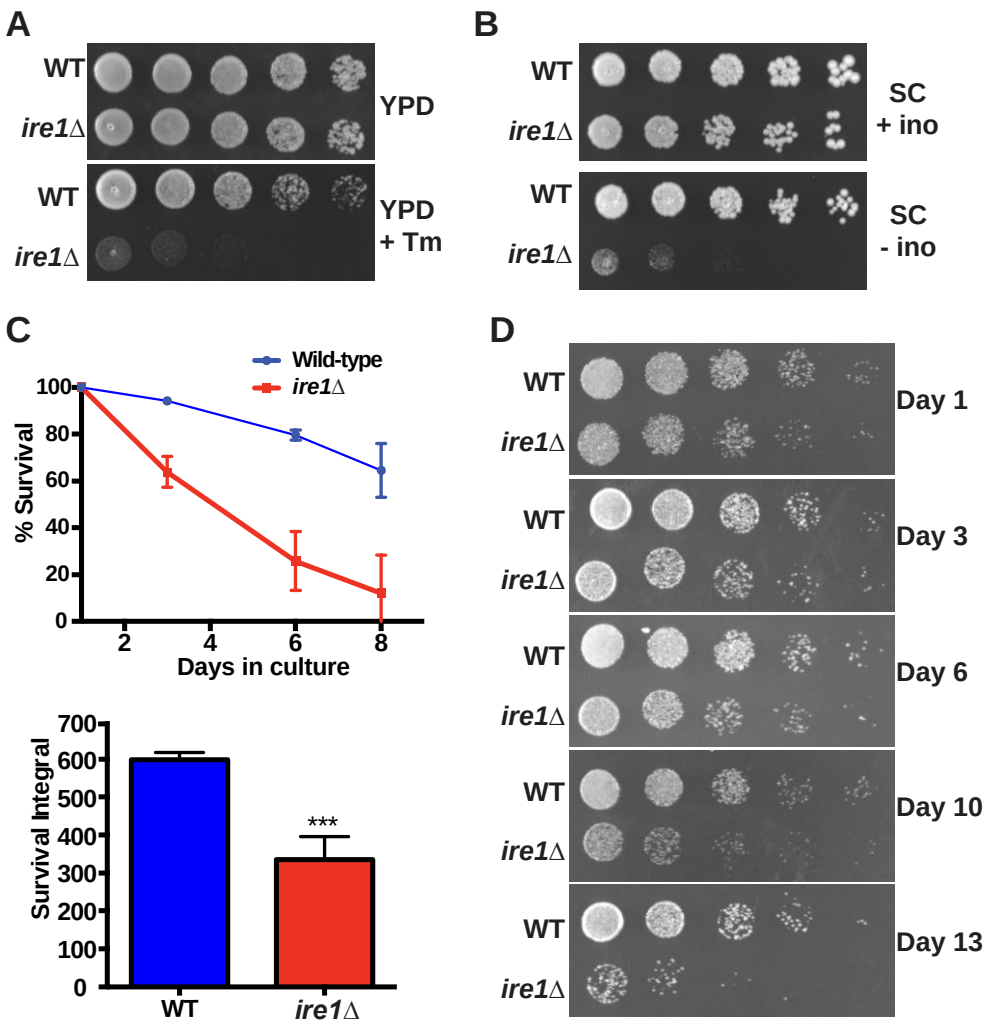

Figure 3.1 Deletion of *IRE1* reduces chronological lifespan. A) Wild-type and *ire1*Δ cells were grown overnight in YPD media, then spotted in serial fivefold dilutions on YPD plates with or without 0.1 µg/ml tunicamycin. Plates were incubated at 30°C for two days. **B)** Wild-type and *ire1*Δ cells were grown overnight in SC media then spotted in serial fivefold dilutions on SC glucose plates with or without inositol. **C)** Wild-type and *ire1*Δ cells were aged in 2% SC glucose media for indicated time periods. Cells were stained with propidium iodide and fluorescence data was analyzed to generate survival curves and survival integrals and statistical analysis was performed using t-tests (n=5, $P < 0.0001$). **D)** Alternatively, cells were spotted in fivefold dilutions on agar and incubated at 30°C for two days before imaging.

While *ire1*Δ cells show decreased viability compared to wild-type during the post-diauxic shift, they still displayed decreased lifespan after reaching stationary phase at day 3, indicating that Ire1 plays a role in both early and later phases of the aging process. These data confirm that *ire1*Δ cells show decreased survival during aging. Overall, these results indicate that Ire1's functions and therefore a functional UPR are indeed essential for CLS, and the composition of the growth media can have important implications for aging studies in yeast (Matecic et al., 2010). Of note, the commonly used SC media recipe for CLS assays contains low levels of inositol (2 μg/ml), usually present in the yeast nitrogen base (YNB) (Hu et al., 2013). We find that *ire1*Δ cells display reduced viability after overnight culturing in SC media (**Supplemental Figure 3.2A**), which may be caused by the need for cells to activate the UPR when cultured in media containing low inositol levels (Moir et al., 2012). Indeed, wild-type cells displayed increased UPR induction when cultured in aging media (standard SC) compared to either YPD or SC supplemented with excess inositol (**Supplemental Figure 3.2B**), as measured with a fluorescent transcriptional reporter containing a red fluorescent protein under a series of 4 tandem UPRE sequences (UPR-mCherry) (Merksamer et al., 2008). *ire1*Δ+UPR-mCherry cells, which lack functional UPR, were used as a negative control. This increased UPR expression in SC compared to YPD or SC + inositol indicates that a lack of inositol in standard SC media, which is frequently used for CLS studies, causes UPR induction. Moreover, inositol synthesis is important for logarithmically growing cells and is repressed at stationary phase (Griac and Henry, 1999), reflecting changing lipid metabolism during the aging process. We indeed observed gradually decreased expression of *INO1* in wild-type cells, the gene encoding the inositol 3-phosphate synthase require for synthesis of phosphatidylinositol (**Supplemental Figure 3.2C**). However, *ire1*Δ cells showed greatly reduced *INO1* expression in log phase compared to wild-type cells (**Supplemental Figure 3.2C**). These data show that media composition, particularly inositol content, significantly affects yeast growth and gene expression, especially in UPR deficient cells. Thus, we supplemented the SC media with 100 μg/ml inositol for all experiments using UPR-compromised cells.

3.2.2 pH buffering of the growth media promotes longevity of UPR-deficient cells

We next investigated which key determinants of CLS are affected in *ire1*Δ cells. We first tested if *ire1*Δ or *hac1*Δ cells (deletion of the transcription factor activated by Ire1 during the UPR) displayed increased sensitivity to acetic acid. Acetic acid is produced by aged yeast; it is also known to correlate with decreased lifespan (Mirisola and Longo, 2012). Cell viability assessment did not show differential sensitivity to acetic acid in *ire1*Δ and *hac1*Δ cells (**Figure 3.2A**). Next, wild-type, *ire1*Δ, and *hac1*Δ cells were grown in pH-buffered SC media (MES 0.1M, pH 6), which has been shown to extend CLS by counteracting the effects of acetic acid accumulation in aging yeast cultures (Burtner et al., 2009b). All three strains showed the same degree of lifespan extension in this media (**Figure 3.2B**). Therefore, our data indicate that buffering acidification of the media antagonizes the toxicity caused by the loss of ER proteostasis during chronological aging.

3.2.3 Caloric restriction antagonizes the loss of UPR functions during aging

Previous studies have shown that caloric restriction extends lifespan in yeast and other models, and that many aging defects are rescued through caloric restriction (Choi et al., 2013; Jiang et al., 2000; Lin et al., 2001). To determine if the protective effect of caloric restriction on aging can function in the absence of a functional UPR, wild-type and *ire1*Δ cells were grown and aged in standard SC media (2% glucose) and in calorie-restricted SC media (0.1% glucose). CLS was measured using propidium iodide staining. CLS was also monitored via spot assays on standard SC agar plates (**Figure 3.3B**). We found that caloric restriction extended lifespan equally in both wild-type and *ire1*Δ cells, indicating that caloric restriction indeed promotes longevity by improving ER homeostasis (**Figure 3.3A, B**). Yeast cells are usually grown in media containing 2% glucose. Under these conditions, cells produce most of their energy via fermentation, and mitochondrial respiration is mostly repressed. When glucose is consumed, and its availability is significantly reduced, cells undergo the diauxic shift and activate respiration. Mitochondrial respiration is therefore essential for CLS, and cells lacking functional mitochondria are short-lived (Aerts et al., 2009; Breitenbach et al., 2012; Demir and Koc,

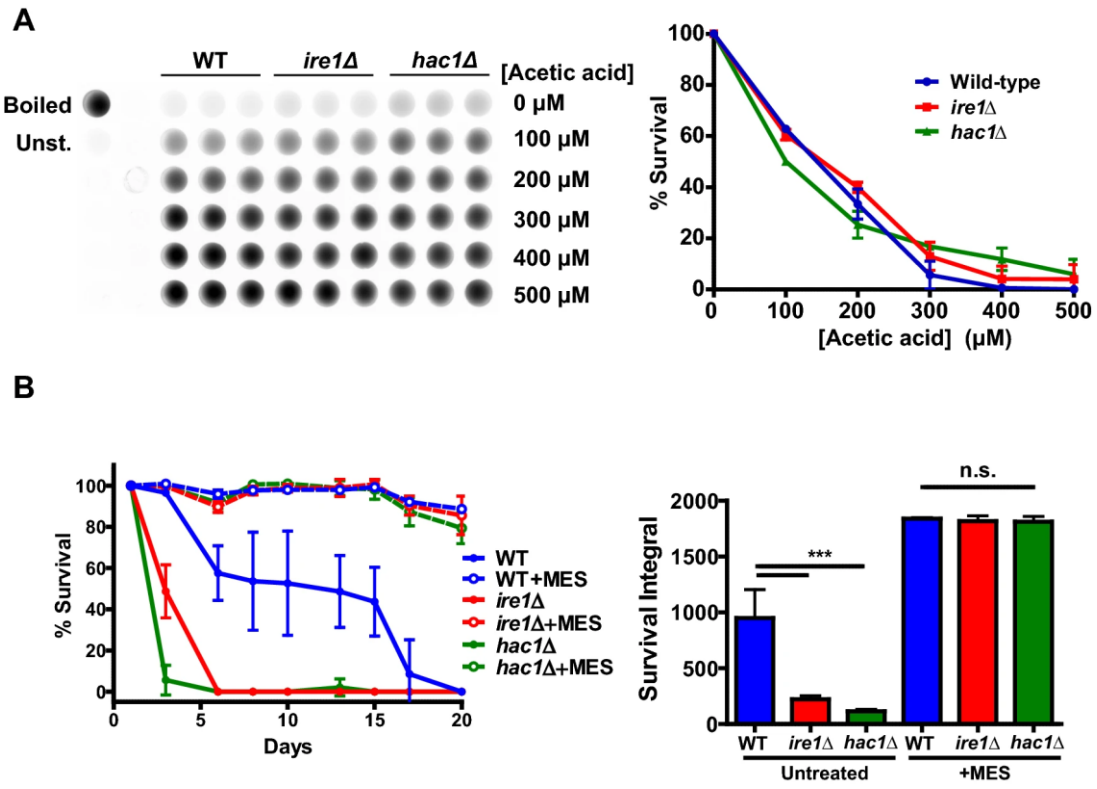

Figure 3.2 Deletion of *IRE1* or *HAC1* does not alter sensitivity to acetic acid treatment or response to pH buffered aging. A) Wild-type, *ire1*Δ, and *hac1*Δ cells were treated with increasing concentrations of acetic acid and stained with propidium iodide (n=3). **B)** Wild-type, *ire1*Δ, and *hac1*Δ cells were aged in standard or pH buffered SC media (MES 0.1M, pH 6). Survival curves and survival integrals were generated from fluorescence using ANALYSR software to quantify differences and statistical analysis was performed using t-tests (n=5, $P = 0.0002$).

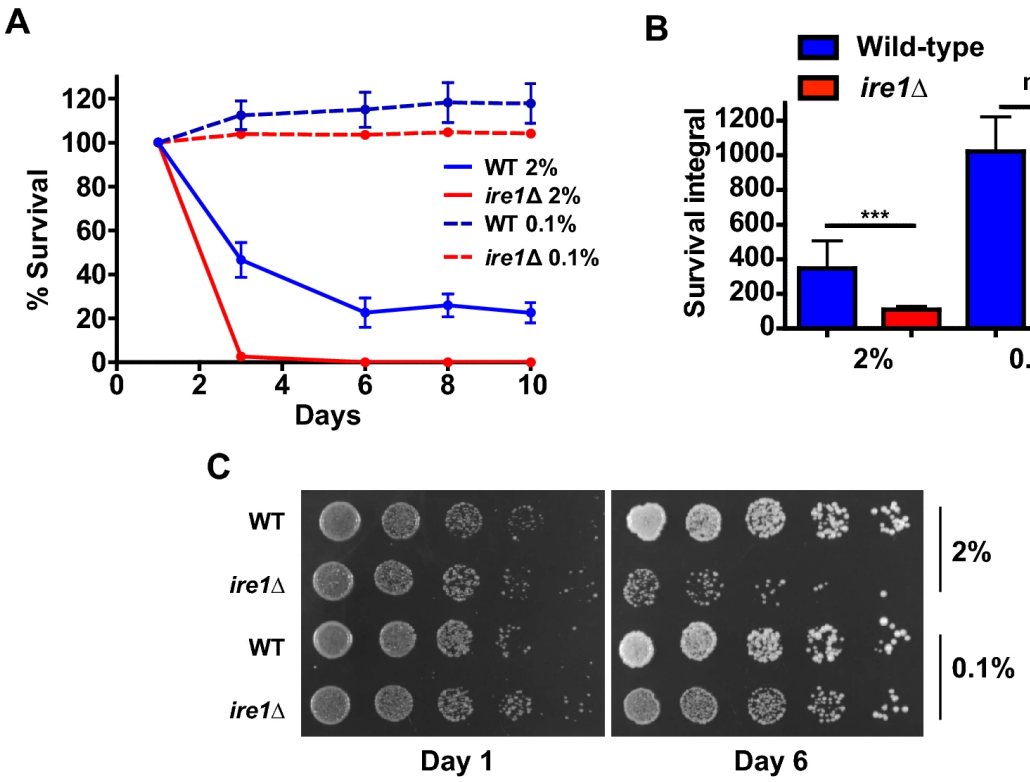

Figure 3.3 Caloric restriction rescues *ire1*Δ cells' aging defect. A) Wild-type or *ire1*Δ cells were aged in either standard 2% glucose media or 0.1% glucose media (calorie restricted). Cells were stained with propidium iodide and fluorescence data was analyzed to generate survival curves and survival integrals, and statistical analysis was performed using t-tests (n=8, P = 0.0002). **B)** Wild-type or *ire1*Δ cells were chronologically aged in either standard 2% glucose SC or 0.1% glucose SC media (calorie restricted), then spotted in serial fivefold dilutions on standard SC agar plates. Plates were incubated at 30°C for two days.

2010; Ruetenik et al., 2016; Smith et al., 2007). To test whether the reduced lifespan observed for *ire1*Δ cells is due to impaired mitochondrial respiration, we compared their growth to wild-type cells in presence of a non-fermentable carbon source, i.e., glycerol, after washing to remove residual glucose (**Supplemental Figure 3.3**). We did not find significant differences in CLS for wild-type versus *ire1*Δ cells grown in glycerol, indicating that mitochondrial respiration does not cause growth defects in *ire1*Δ cells.

3.2.4 UPR-mediated *KAR2* upregulation is required for chronological aging

Because we found that Ire1 is required for chronological aging, we next investigated how UPR activation affects aging. By comparing the cellular response to ER stress at day 0 and day 4 of aging (a point at which defects in proteostasis and decreased viability have been previously shown to occur (Fabrizio and Longo, 2007)), we tested whether aging and ER stress could cause compounding effects on UPR signaling. Using a transcriptional reporter consisting of a LacZ gene under the control of a promoter carrying 4 tandem UPR response elements (UPRE), we found that wild-type cells demonstrated a significant increase in UPR signaling during aging. This increase was exacerbated when combined with tunicamycin treatment. Aged cells undergoing ER stress activated the UPR to a much greater extent than did younger cells (**Figure 3.4A**). We also tested the sensitivity of young vs. aged cells to tunicamycin-induced stress as measured by colony forming unit (CFU) assay. In agreement with our observation that UPR is activated in aging cells, we found aged cells displayed increased resistance to tunicamycin when compared to exponentially growing cells (day 0) (**Figure 3.4B**). Based on these data, we explored whether specific UPR targets are required for CLS.

We next examined the role of Kar2 during aging. Kar2 is the yeast homologue of mammalian BiP, a chaperone protein found in the ER that dissociates from Ire1 with increased misfolded protein burden, thus allowing Ire1 to activate the UPR (Bertolotti et al., 2000; Kimata et al., 2003; Pincus et al., 2010). Kar2 levels are increased during the UPR in order to decrease the unfolded protein levels in the ER lumen (Kohno et al., 1993; Mori et al., 1992). In the absence of UPR activation, however, *KAR2* transcription

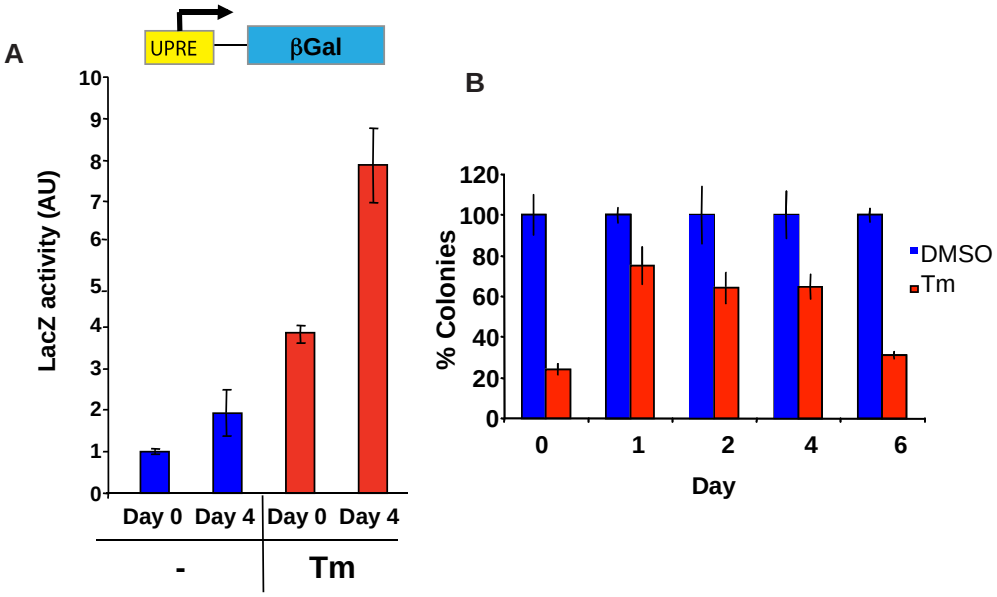

Figure 3.4 The UPR is upregulated during aging. A) UPRE-LacZ reporter system was used to reveal baseline UPR activation in cells grown at mid-log (day 0) and stationary phase (4 days) compared to those treated with tunicamycin, an ER stressor. Yeast cells were incubated for the indicated times in liquid medium and then exposed to ER stress (5 µg/ml tunicamycin (Tm) for 1 h). Equal numbers of cells from liquid cultures were assayed for induction of the βGal reporter protein. βGal activity at day 0 without Tm treatment was set as 1 and the other values were calculated accordingly. The average and standard deviations (error bars) of three independent experiments are shown. **B)** Yeast cells were incubated for the indicated times in liquid medium and then either exposed to ER stress (5 µg/ml tunicamycin for 1 h, red columns) or with DMSO (blue columns). Equal number of cells from the liquid cultures were then spread on plates containing rich media (YPD) and incubated for 2-3 days. The numbers of colony forming units (CFUs) were counted and the number of colonies formed from the untreated culture was set at 100%. Viability of the cells was not reduced even by the prolonged period of aging (4 and 6 days). The average and standard deviations (error bars) of three independent experiments are shown.

still occurs, albeit at a lower rate (Kimata et al., 2003; Mori et al., 1992). Because of its well-defined role in resolving ER stress, and because of the link between unresolved ER stress and shortened CLS, we hypothesized that it may also play a role in normal CLS. Interestingly, *KAR2* expression is downregulated under conditions of caloric restriction (Choi et al., 2013). We thus asked if baseline, i.e. non-induced, *KAR2* transcription levels are sufficient to respond to CLS-induced ER stress or if UPR-dependent induction is required. To this end, we took advantage of a *KAR2* mutant strain that carries a defective UPRE sequence in its promoter (replaced with a non-specific sequence), rendering the *KAR2* gene insensitive to UPR activation (*KAR2-upre*^{d}), while baseline *KAR2* expression levels remain unaffected (Hsu et al., 2012). *KAR2-upre*^{d} cells displayed increased sensitivity to ER stress induced by tunicamycin but not DTT (Hsu et al., 2012) (**Figure 3.5A**). These data indicate that *KAR2* upregulation is required for specific UPR-inducing stresses. Next, we investigated the requirement for *KAR2* upregulation in CLS. *KAR2-upre*^{d} and control *KAR2*-UPRE wild-type cells were grown and aged in SC media. At defined time points during aging, cells were spotted on agar plates and assessed for growth defects. We found that *KAR2-upre*^{d} cells had a shortened CLS, which was obvious as early as three days into the aging process (**Figure 3.5B**). To confirm these results, the same aging experiment was conducted using propidium iodide staining. Again, *KAR2-upre*^{d} showed a significantly reduced CLS (**Figure 3.5C**). Finally, to further assess whether Kar2 abundance is required for CLS, the lifespan of wild-type cells was compared to that of those expressing the *KAR2*-DAmP allele (Decreased Abundance by mRNA Perturbation) (Breslow et al., 2008), which causes overall decreased Kar2 expression levels (and therefore activity) without deleting the gene entirely, allowing the investigation of the effects of essential genes (Breslow et al., 2008). It should be noted that both the *KAR2*-DAmP cells and their controls are expressed in a longer-lived BY4741 background as opposed to the W303 strains used in other experiments. This allele causes no significant growth defect in the logarithmic growth phase with a median growth rate of 1.013 compared to wild-type (Breslow et al., 2008), yet the lifespan of the *KAR2*-DAmP cells was significantly reduced compared to that of wild-type cells (**Figure**

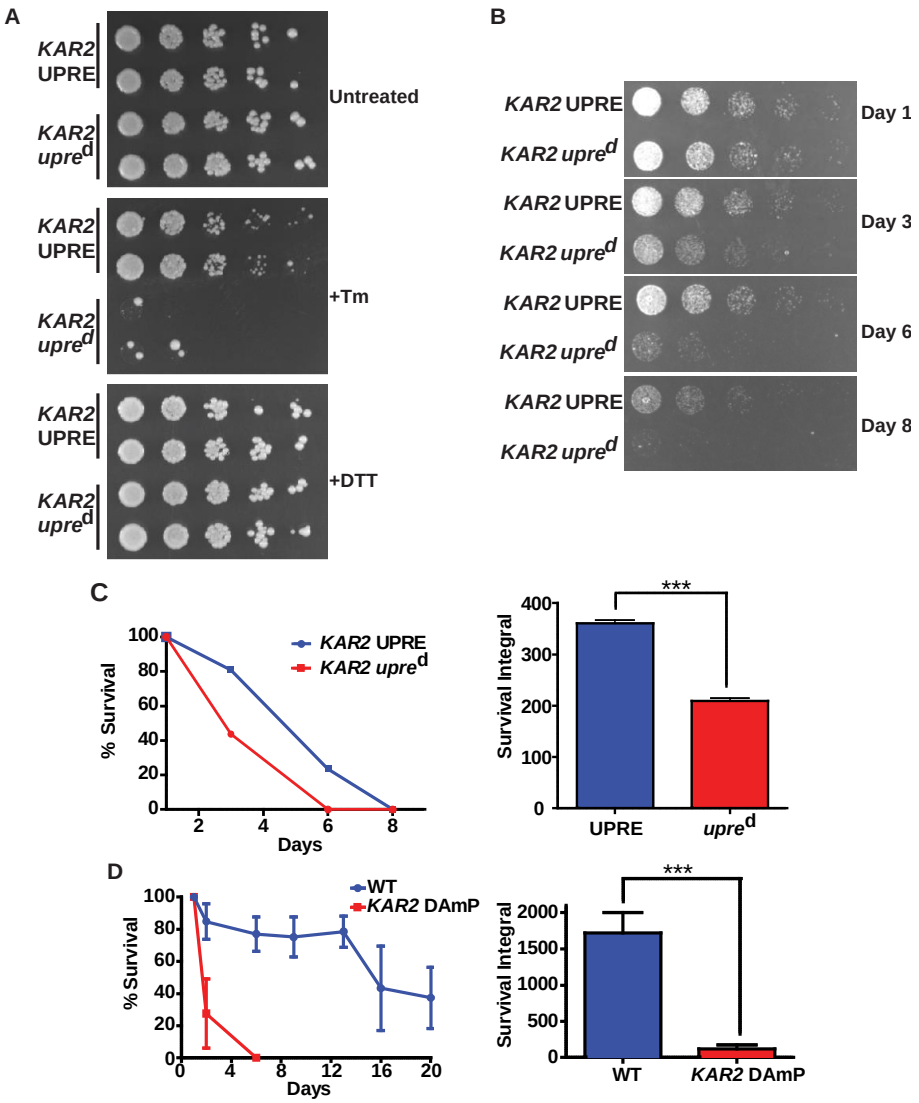

Figure 3.5 UPR-dependent upregulation of *KAR2* regulates chronological aging. A) *KAR2*-UPRE wt or *KAR2-upre*^{d} expressing cells were spotted on YPD plates containing either tunicamycin or DTT. **B)** *KAR2*-UPRE wt or *KAR2-upre*^{d} expressing cells were chronologically aged in SC media and spotted in serial fivefold dilutions over the course of aging. **C)** *KAR2*-UPRE wt or *KAR2-upre*^{d} expressing cells were chronologically aged in SC media and stained with propidium iodide at various time points. Fluorescence was assessed to generate survival curves and survival integrals. Statistical analysis was performed using t-tests. (n=5, $P < 0.0001$) **D)** Wild-type cells and cells expressing a *KAR2* DAmP allele were aged in SC media and stained with propidium iodide at various time points. Fluorescence was assessed to generate survival curves and survival integrals. Statistical analysis was performed using t-tests. (n=3, $P = 0.0006$).

3.5D). This further confirms that proper *KAR2* regulation by the UPR and proper Kar2 levels are required for chronological aging in *S. cerevisiae.*

3.2.5 Impaired ERAD decreases lifespan

ERAD relieves ER stress by reducing misfolded protein burden in the ER through targeting misfolded proteins for retrotranslocation to the cytosol followed by their degradation by the ubiquitin proteasome system. Via its regulation of Ire1, Kar2 indirectly modulates the expression of the UPR genes encoding proteins involved in ER-associated degradation (ERAD) (Hsu et al., 2012; Kabani et al., 2003). Since ERAD-defective *KAR2-upre*^{d} cells (Hsu et al., 2012) are short-lived (**Figure 3.5**) and chronologically aged cells show decreased ability to degrade ERAD substrates (Cohen et al., 2016), we hypothesized that genes encoding components of the ERAD machinery are required for CLS. Misfolded proteins accumulate during aging due to decreased efficiency of ERAD, possibly mediated by the UPR (Cohen et al., 2016). Depending on the location of the misfolded protein's degradation tag (degron), the protein is recognized by different branches of ERAD. Specifically, the E3 ubiquitin ligase complexes that recognize the proteins differ: Hrd1 recognizes proteins with luminal or membrane-oriented degrons (ERAD-L or M), whereas Doa10 recognizes proteins with cytoplasmic degrons (ERAD-C) (Carvalho et al., 2006; Rubenstein et al., 2012). We tested both E3 ligases to determine their effects on chronological lifespan. To this end, we generated deletion mutants of either *HRD1* or *DOA10* and performed CLS assays. Surprisingly, neither deletion mutant showed reduced CLS compared to wild-type cells, and actually showed a slight lifespan extension (**Figure 3.6A**, **B, C**). By contrast, a double deletion mutant (*hrd1*Δ *doa10*Δ) decreased CLS (**Figure 3.6D**), indicating a functional overlap of the two ERAD branches during the aging process and that a minimal level of functional ERAD activity is required for chronological aging.

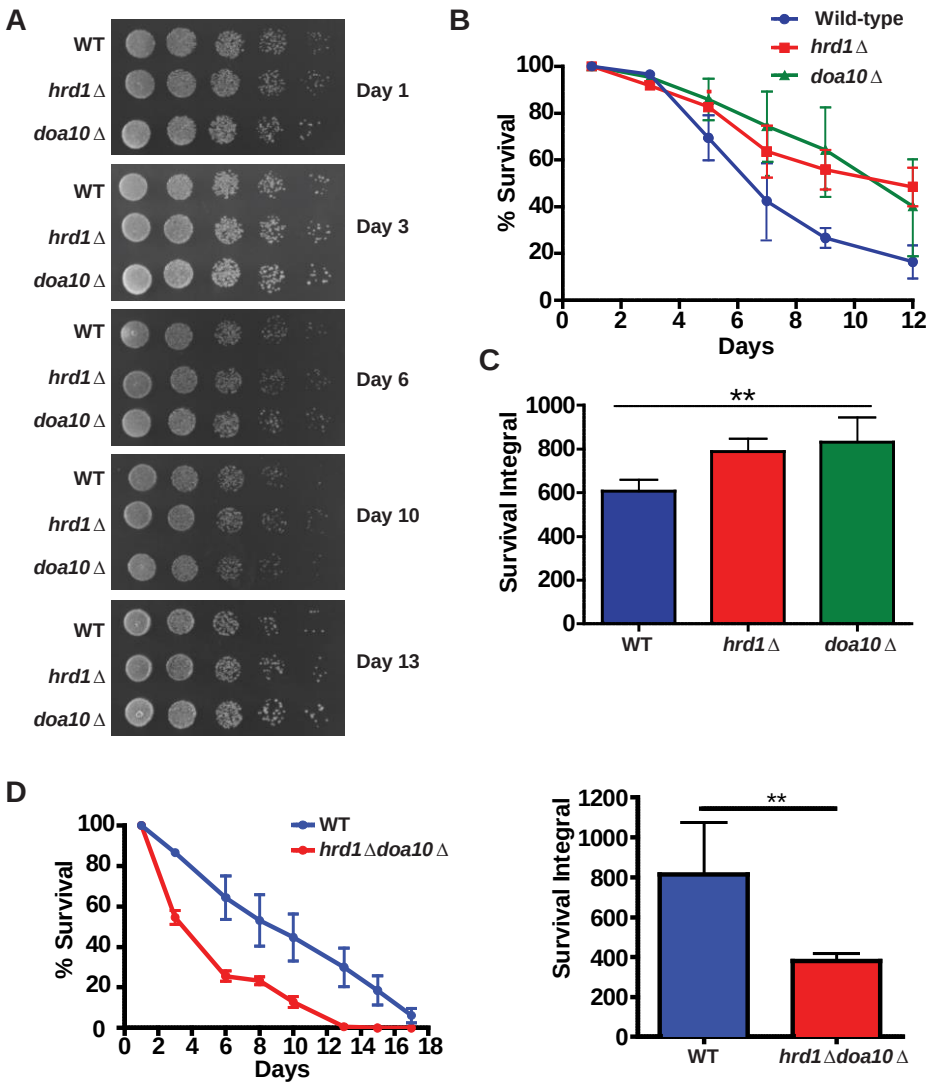

Figure 3.6: ERAD function impacts chronological aging. A) Wild-type cells and cells harboring deletions of *HRD1* or *DOA10* were aged in SC media for the indicated time points. Cells were spotted on YPD plates over the course of the aging process. **B/C)** Wild-type cells and cells harboring deletions of *HRD1* or *DOA10* were aged in SC media and stained with propidium iodide at each time point. Fluorescent data were assessed to generate survival curves and survival integrals and statistical analysis was performed using t-tests (n=5, WT vs *doa10*Δ $P = 0.0037$, WT vs *hrd1*Δ $P = 0.008$). **D)** Wild-type cells and *hrd1*Δ *doa10*Δ cells were aged in SC media and stained with propidium iodide at each time point. Fluorescent data were assessed to generate survival curves and survival integrals (n=5, $P = 0.0063$).

3.3 Discussion

Previous studies have shown that deletion of *IRE1* does not affect replicative lifespan (Labunskyy et al., 2014), nor does it prevent replicative lifespan extension via other means (e.g. CR or nitrogen starvation) (Postnikoff et al., 2017). This may be attributable to the fact that ER stress itself can extend replicative lifespan and does so independently of Ire1, possibly through increasing basal levels of autophagy and reducing global protein translation (Ghavidel et al., 2015; Shen et al., 2019). Differential requirement for Ire1 between the two aging paradigms in yeast highlights major differences between the role of ER protein homeostasis in replicative versus chronological aging. Similarly, the pH of the growth media is a major regulator of CLS (Burtner et al., 2009b) but has no significant effect on replicative aging (Wasko et al., 2013). One simple explanation to the reduced chronological lifespan of *ire1*Δ cells would be the need for UPR activation upon glucose starvation. Previous studies show that under normal growth conditions (2% glucose), glucose is depleted from the growth media after 12h. Depletion is exacerbated during caloric restriction conditions (~6h) (Burtner et al., 2009b). Glucose starvation is known to lead to defects in glycosylation that results in UPR activation in higher eukaryotes (Kozutsumi et al., 1988; Shiu et al., 1977). Our data showing that caloric restriction extends lifespan of *ire1*Δ cells argue against this. Similarly, transcriptional profiling of caloric restricted cells shows decreased expression of genes linked to ER stress (Choi et al., 2013). Our data suggest that Ire1 is not required for this form of CLS extension. This is interesting considering that deletion of *SIR2* prevents caloric restriction-mediated lifespan extension in yeast (Lin et al., 2000) and that Sir2.1 regulates the response to ER stress in worms (Viswanathan et al., 2005). This may be explained by the fact that Sir2 in yeast is also linked to another stress response pathway, the Heat Shock Response (HSR) (Weindling and Bar-Nun, 2015), which has been shown to alleviate ER stress and has been linked to mitochondrial PQC (Koike et al., 2018; Liu and Chang, 2008). HSR functionality may thus be more important than the UPR in this specific form of lifespan extension.

Unexpectedly, both wild-type and UPR deletion mutants displayed similar sensitivity in the presence of acetic acid, a natural byproduct of yeast aging that has been suggested to

contribute heavily to yeast cell death during chronological aging (Burtner et al., 2009b). However, we observed extended lifespan when both wild-type and *ire1*Δ cells were aged in pH-buffered media, which extends CLS by counteracting the effects of media acidification in aging yeast cultures (Burtner et al., 2009b). These data suggest that while media acidification contributes to CLS, decreased lifespan observed in *ire1*Δ cells is unlikely to be due to an increased sensitivity to the acetic acid produced during aging. Burtner et al. concluded that pH buffering may extend CLS by reducing the oxidative and acid stress caused by the natural byproducts of yeast fermentation (acetic acid and reactive oxygen species). Both of these stressors are resolved via PQC mechanisms that are not directly regulated by the UPR. This supports our data showing that wild-type and *ire1*Δ cells show similar phenotypes during both acetic acid treatment and pH buffering. This is also consistent with our data wherein we demonstrate that *ire1*Δ cells display no growth defect when switched to a non-fermentable carbon source (glycerol). However, acetic acid is known to induce the UPR in yeast and acetic acid-induced UPR is also exacerbated in presence of ethanol (Kawazoe et al., 2017), which is another determinant of cell toxicity in CLS (Fabrizio et al., 2005). One could envisage that the environment created by chronologically aged cells exposes cells with a combination of multiple stresses (acid, ethanol, nutrient starvation) that ultimately affect ER homeostasis among other PQC pathways. Moreover, UPR can be activated in absence of misfolded proteins, via changes in the lipid composition of the ER membrane (Kono et al., 2017; Lajoie et al., 2012; Pineau et al., 2009; Promlek et al., 2011; Volmer et al., 2013) and sphingolipids have been shown to play a role in yeast RLS (Singh and Li, 2018). Additionally, age-related changes in the lipidome have been detected in *C. elegans* (Wan et al., 2019). How changes in the yeast lipidome regulate UPR activation during the aging process remains unclear.

Our study supports a crucial role for ERAD in chronological aging. We observed that cells carrying deletions for both *HRD1* and *DOA10* displayed decreased longevity. This mutant had shortened CLS compared to both wild-type cells and single deletion mutants, which is consistent with literature demonstrating that deletion of either *CUE1* or *UBC6*, which regulate both the Hrd1 and Doa10 complexes' function (Biederer et al., 1997),

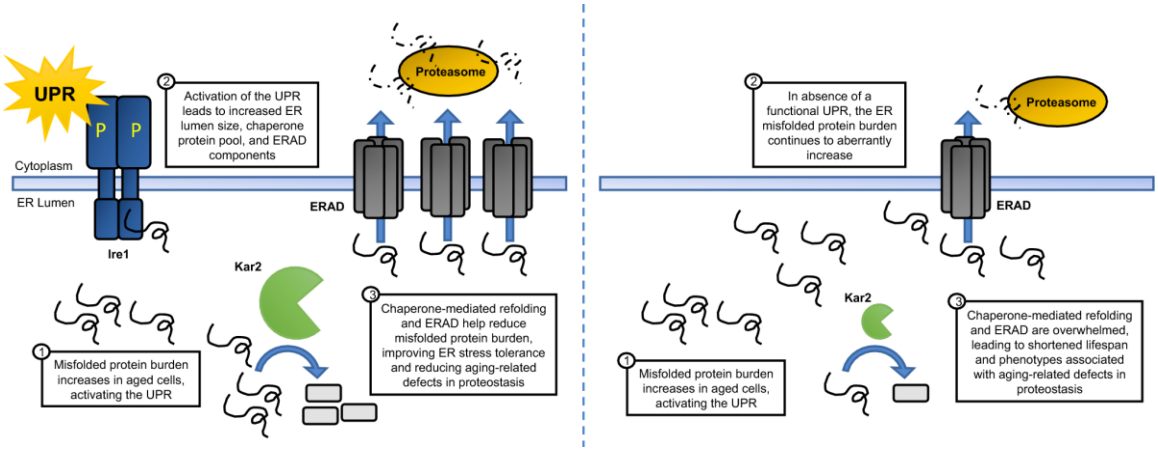

Figure 3.7 The role of functional UPR during aging. As misfolded protein burden increases in aging cells, activation of the UPR allows the cell to respond and resolve ER stress by expanding the ER lumen, increasing translation of chaperone proteins such as Kar2, and increasing translation of ERAD machinery, thereby preventing the accumulation of misfolded proteins. Cells lacking functional UPR are unable to respond to this increase in misfolded proteins in the ER lumen, causing further ER stress that the cell cannot efficiently resolve. As normal PQC machinery becomes overwhelmed, cells display shortened chronological lifespan and other age-related phenotypes caused by defects in proteostasis, such as sensitivity to other ER stressors.

causes shortened CLS as evaluated by competition-based growth assays (Garay et al., 2014). These results point to redundancy between the Hrd1 and Doa10 pathways that may allow maintenance of the ER misfolded protein burden below the threshold that might result in shortened CLS. Indeed, it has been shown that the ERAD substrate Sec62-DEG1 is not fully stabilized in either *hrd1*Δ or *doa10*Δ cells, and that the mutant form of Sec62-DEG1, which cannot properly bind to Sec63, exhibits the same lack of complete stabilization. In both cases, the ERAD substrate is stabilized by double deletion of *hrd1*Δ and *doa10*Δ (Rubenstein et al., 2012). In addition, Sec62-DEG1 is stabilized by the deletion of *UBC7*, an E2 ubiquitin conjugating enzyme that is upstream of both the Hrd1 and Doa10 pathways (Mayer et al., 1998; Rubenstein et al., 2012). Finally, compromised ERAD in the *KAR2 upre*^{d} strains (Hsu et al., 2012) could, at least in part, explain why *KAR2* upregulation by the UPR is critical for CLS. Yet, surprisingly, deletion of either of the ERAD E3 ubiquitin ligases alone did not impair chronological aging. This contrasts with previous results showing that deletion of *HRD1* results in reduced lifespan (Matecic et al., 2010). The source of this discrepancy may reflect properties of the different strain backgrounds employed (W303 vs BY4741). The simplest explanation for this CLS extension could be that deletion of ERAD components activates the UPR (Jonikas et al., 2009; Travers et al., 2000) and that UPR activation confers stress resistance along the aging process, as proposed by others (Labunskyy et al., 2014). Indeed, increased ERAD activity by the UPR is required for ER stress tolerance (Friedlander et al., 2000). Further, the need for UPR activation in ERAD-compromised cells is revealed by the synthetic lethal phenotype seen in cells with deletions in both *IRE1* and genes encoding ERAD components in the W303 background used in our study (Travers et al., 2000). A similar role for ERAD has observed in previous studies on RLS as well, where deletion of some UPR targets extended RLS when upstream UPR signaling (via Ire1 and Hac1) was intact (Labunskyy et al., 2014). Taken together, these data indicate that UPR and ERAD signaling work in tandem during stress adaptation to promote longevity. Interestingly, in worms, lifespan extension by addition of N-acetylglucosamine is associated with increased ERAD activity (Denzel et al., 2014) and addition of N-acetylglucosamine as well as adaptive aneuploidy (causing duplication of ER stress-responsive genes including ERAD targets) have also been shown to rescue UPR impairment in yeast (Beaupere and

Labunskyy, 2019; Beaupere et al., 2018). Similarly, ERAD can protect against age-mediated physiological decline in flies (Sekiya et al., 2017), supporting a role for active ERAD in aging across organisms. Finally, loss of BiP/Kar2 function is linked to the appearance of motor disabilities during aging in mice (Jin et al., 2014), supporting a critical role for Kar2/BiP during the aging process.

Our study establishes a clear link between UPR activation and CLS in yeast (**Figure 3.7**) that could guide further investigation of experimental paradigms such as age-dependent protein misfolding disease models, including Parkinson's and Huntington's disease, wherein UPR activation and protein misfolding play a role in the disease onset (Di Gregorio and Duennwald, 2018; Jiang et al., 2016; Klaips et al., 2018; Martínez et al., 2017; Mercado et al., 2018; Ruegsegger and Saxena, 2016; Scheper and Hoozemans, 2015; Vidal and Hetz, 2012; Vidal et al., 2012). The breadth of the cellular functions affected by the UPR suggests that many more signaling nexuses regulating ER stress tolerance are crucial for the aging process and remain to be investigated. Among these, it will be key to decipher how UPR signaling integrates with other lifespan regulators such as the TOR pathway, which is involved in nutrient sensing and regulates autophagy, (Fabrizio et al., 2001; Johnson et al., 2013; Schieke and Finkel, 2007), and the heat shock response, which resolves misfolded proteins in the cytosol (Baird et al., 2014; Douglas et al., 2015), to determine how stress signaling modulates lifespan under both normal and disease states.

3.4 Materials and Methods

3.4.1 Strains and Plasmids

All strains were derivatives of W303 or BY4741 and are listed in **Table 3.1**. The UPR-mCherry (Merksamer et al., 2008) reporter was previously described and obtained from Addgene (#20132). The UPR-lacZ reporter carrying the 4X UPRE sequences inserted upstream of the disabled *CYC1* promoter-driven *lacZ* reporter gene fusion was previously described (Cox and Walter, 1996).

Table 3.1: Strains used in chapter 3

Strain	**Description**	**References**
YDP001	W303 *leu2-3,112 trp1-1 ura3-1 his3-11,15 mat a*	(Pincus et al., 2010)
YDP002	Same as YDP001 except *ire1*Δ: KAN	
YSC001	Same as YDP001 except *hrd1*Δ: NAT	This study
YSC002	Same as YDP001 except *doa10*Δ: NAT	This study
YSC003	Same as YDP001 except *hrd1*Δ:: KAN, *doa10*Δ: NAT	This study
CHY438	ura3-52:UPRE-KAR2-URA3, kar2::KanMX, W303a background	(Hsu et al., 2012)
CHY220	ura3-52:upred -KAR2-URA3, kar2::KanMX, W303a background	
BY4741	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mat a*	(Brachmann et al., 1998)
KAR2-DAmP	Same as BY4741 except *KAR2*-DAmP:: KAN	(Breslow et al., 2008)

3.4.2 Cell culture and Chronological Lifespan Assays

For every experiment, W303a or BY4741 *S. cerevisiae* cells and their derivatives (see **Table 3.1**) were thawed from frozen glycerol stocks and grown on solid YPD (yeast extract peptone dextrose) media for 2 days at 30°C before being transferred to liquid cultures. A total of 5 mL of liquid cultures were grown to saturation overnight before being separated into replicates (100 µL of overnight cultures in 5 mL of liquid media). All aging experiments were carried out using synthetic complete media (SC). SC and SC +inositol media contained either 2 mg/L or 100 mg/L, respectively. pH buffered aging

was conducted by adding MES buffer (pH 6) to SC media with a final concentration of 0.1M before inoculating with overnight cultures as previously described (Burtner et al., 2009b). Replicates were incubated in polystyrene snap cap tubes at 30°C in a rotating drum over the course of the aging process.

3.4.3 Cell viability staining

Cells were stained with propidium iodide to detect viability as previously described (Chadwick et al., 2016). A total of 200 μL of each cell culture were added to 1.5 mL tubes, plus two extra samples to serve as positive and negative controls. The positive control was boiled for 10 min at 100°C. All samples were pelleted for 2.5 min. All samples except the negative control were resuspended in 200 μL of phosphate-buffered saline (PBS) containing a 1 in 200 dilution of a PI stock solution (1 mg/mL in H_2O). The negative control was resuspended in 200 μL of PBS alone. The samples were incubated at room temperature for 10 min. Imaging was then performed using the Gel Doc system (BioRad) using the red fluorescent filter. The optical density (OD_{600}) of each sample was determined with either plate reader, or by measuring the absorbance of diluted samples in cuvettes. Survival rates and integrals were calculated using the ANALYSR program as previously described (Chadwick et al., 2016). Experiments were ended when percent survival reached zero, or when apparent suppressor strains arose in the populations (e.g. when survival dropped below 20% before increasing again, suggesting the emergence of adaptive mutations).

3.4.4 Spot assays

Aliquots of cell cultures were diluted to OD_{600} 0.2, then serially 5x diluted. Dilutions were then spotted onto YPD or SC agar plates and incubated at 30°C for two days before imaging using the BioRad Gel Doc system.

3.4.5 Fluorescent Microscopy

Aliquots of cells expressing fluorescent proteins were pelleted, resuspended in PBS, and diluted 1000x. 200 μL of each diluted culture was loaded into LABTEK (Nunc Inc.) imaging chambers. Images were acquired using a Zeiss Axiovert A1 wide-field

fluorescent microscope equipped with 560 nm excitation/630 emission (TexasRed) and a 470 nm excitation/525 nm emission (GFP) filters and a 63× 1.4 numerical aperture oil objective and an AxioCam ICm1 R1 CCD camera (Carl Zeiss inc.). Images were analyzed using ImageJ (Schindelin et al., 2015).

3.4.6 LacZ reporter system

β-galactosidase activity was determined as previously described (Duennwald and Lindquist, 2008). Briefly, cells were aged for CLS assays, lysed using a commercial kit (Galacto-Light Kit, Thermo), and luminescence was analyzed with plate reader according to the manufacturer instructions.

3.4.7 RT-qPCR

RNA extraction was performed using MasterPure Yeast RNA Purification Kit (Lucigen). cDNA synthesis was done by qScript Flex cDNA Synthesis Kit (Quanta Bioscience). The cDNA preparations were used as the template for amplification using PerfeCTa SYBR-Green Supermix (Quanta Bioscience). The primers used were *INO1*F: TGCACGTACAAGGACAACGA, *INO1*R: GGCCACTAAAGTGGAGCCAT, *U3*F:cCCCAGAGTGAGAAACCGAAA *U3*R: AGGATGGGTCAAGATCATCG. The relative expression level was calculated using the comparative Ct method and U3 was used as a reference gene.

3.5 Supplemental Figures

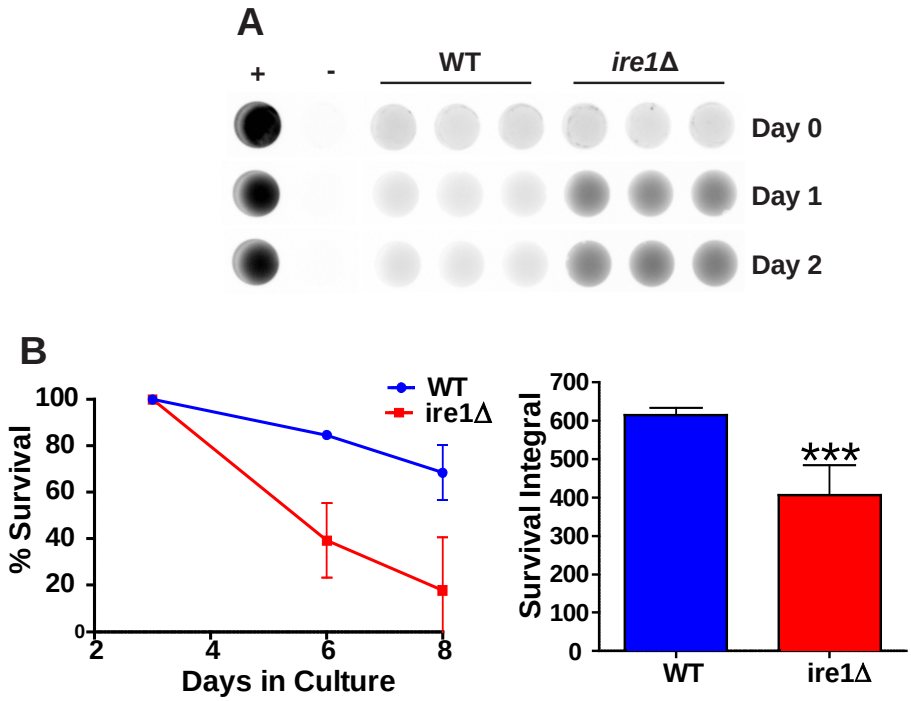

Figure S3.1 Deletion of *IRE1* shortens chronological lifespan independently of the post-diauxic shift. **A)** Wild-type and *ire1*Δ cells were grown in SC media for 8h (day 0), 1 day, and 2 days. Cells were stained with propidium iodide and imaged with a UV trans-illuminator to view fluorescence. Boiled cells were used as a positive control, unstained cells were used as a negative control (n=3). **B)** Wild-type and *ire1*Δ cells were aged in 2% SC glucose media for indicated time periods. Cells were stained with propidium iodide and fluorescence data was analyzed to generate survival curves and survival integrals with day 3 set to 100% survival (as opposed to day 1) and statistical analysis was performed using t-tests (n=5, $P = 0.0004$).

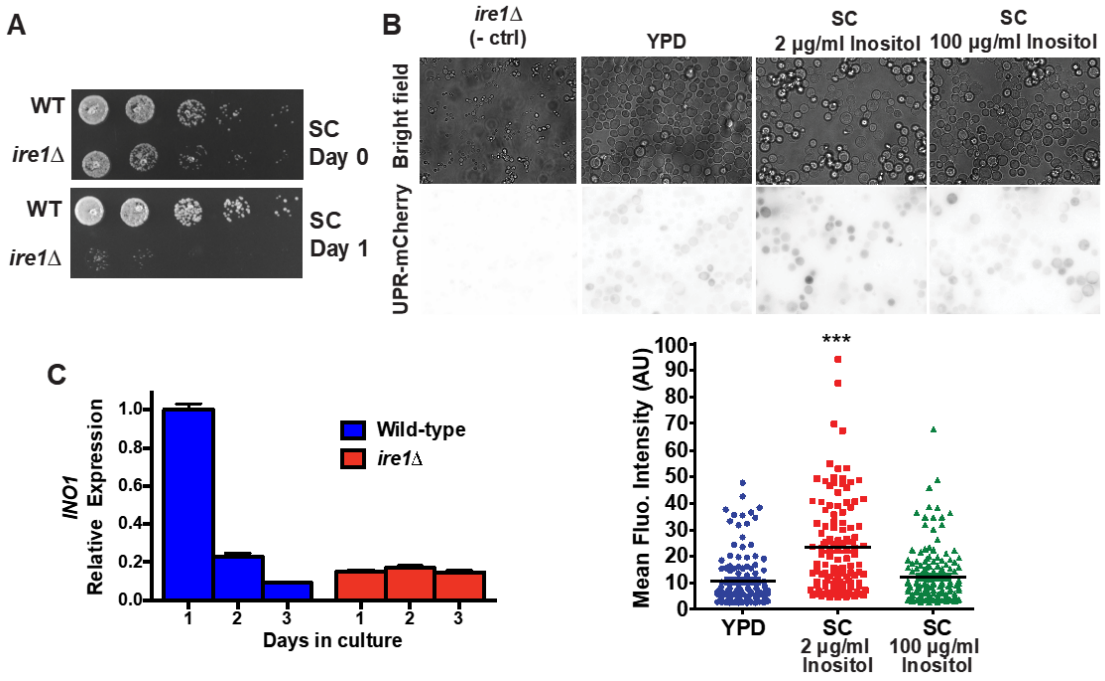

Figure S3.2 Growth media inositol concentration affects cell growth, UPR activation, and gene expression. **A)** Wild-type and *ire1*Δ cells were grown for either 8 hours (Day 0) or overnight (Day 1) in SC media then spotted in serial fivefold dilutions on SC glucose plates. **B)** Cells expressing the fluorescent protein UPR-mCherry were grown overnight in the indicated media, and then analyzed by fluorescent microscopy. Relative fluorescence from individual cells was quantified and plotted, and statistically analyzed using t-tests (n=122-145, $P < 0.0001$). *ire1*Δ+UPR-mCherry is used as a negative control. **C)** Wild-type and *ire1*Δ cells were aged for 1-3 days, then RNA was isolated, reverse transcribed to cDNA, then *INO1* expression was quantified using RT-qPCR.

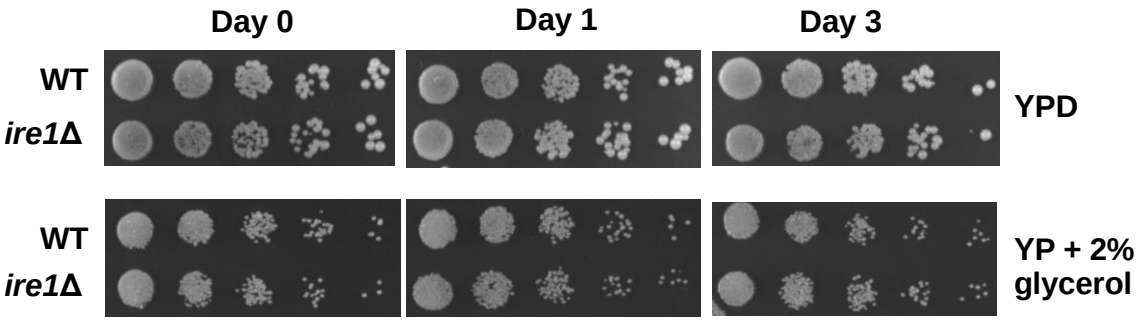

Figure S3.3 Aging defects seen with deletion of *IRE1* are not attributable to defects in mitochondrial respiration. Aged wild-type and *ire1*Δ cells were spotted on YPD or YPG (2% glycerol) plates to assess the effect of switching the cells from fermentation to mitochondrial respiration.

3.6 Acknowledgements

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Chapter 4

4 Regulation of ER stress resistance by tauroursodeoxycholic acid in yeast

ER stress and UPR induction have been repeatedly linked to human disease. As a result, numerous attempts have been made to modulate these. TUDCA, a bile acid, has been shown to reduce ER stress in some aging- and ER stress-related disease models, potentially by acting as a chemical chaperone. Using insights into the UPR signaling pathway components developed in Chapter 3, this chapter aimed to identify TUDCA's mechanism of action and impact on the UPR, and to determine whether TUDCA is able to function as a chemical chaperone as proposed in the literature.

4.1 Introduction

The accumulation of misfolded proteins in the endoplasmic reticulum, either due to defects in protein folding or quality control mechanisms such as endoplasmic reticulum associated degradation (ERAD), generates a condition known as ER stress. ER stress is a common element of numerous diseases, including neurodegenerative diseases like Alzheimer's or Parkinson's, as well as chronic conditions like diabetes and inflammation (Cooper et al., 2006; Engin et al., 2013; Haynes et al., 2004; Hotamisligil, 2010; Lee et al., 2010; Ozcan et al., 2004). Long-term, unresolved ER stress can lead to cell death through several mechanisms, exacerbating disease phenotypes (Sano and Reed, 2013). Because of its ubiquity, targeting or modulating ER stress could be an effective way to reduce the severity of its associated diseases. ER stress could (theoretically) be ameliorated by directly reducing misfolded protein burden (either by refolding or degrading misfolded/unfolded proteins) or circumvented by targeting its downstream signaling pathways. One compound that shows promise in reducing ER stress for therapeutic purposes is tauroursodeoxycholic acid (TUDCA).

TUDCA is a taurine-conjugated bile acid produced in small amounts in the human body by intestinal bacteria. It is the primary bile acid produced in Asian and North American black bears (Boatright et al., 2009). TUDCA (and bear bile acid in general) has been used for centuries as a traditional Chinese remedy and has been recently shown to improve symptoms and slow progression in numerous conditions (Boatright et al., 2009; Keene et al., 2002; Nunes et al., 2012; Rivard et al., 2007). TUDCA has been identified as a potential cytoprotective treatment in conditions such as retinal degeneration (Drack et al., 2012), Alzheimer's disease (Nunes et al., 2012), spinal cord injury (Kim et al., 2018), Huntington's disease (Keene et al., 2002), and many others (Vang et al., 2014). One major commonality between the diseases improved by TUDCA is their link to ER stress, protein misfolding, and dysregulation of a major stress response pathway normally activated in response to ER stress: the unfolded protein response (UPR). Despite promising research on TUDCA in all of these fields, its exact mechanism of action is still unclear; it affects a variety of signaling pathways and has numerous targets (Vang et al., 2014; Xie et al., 2002). TUDCA has been previously hypothesized to act as a chemical

chaperone, a category of small molecules that are able to directly improve protein folding, stability, or trafficking. TUDCA is theorized to modulate UPR activation by stabilizing protein conformation, thereby improving the folding capacity of the ER and reducing ER stress (Omura et al., 2013; Ozcan et al., 2006).

In a study on pulmonary fibrosis, TUDCA was found to decrease UPR signaling by decreasing activation of ATF6 and eIF2α (Omura et al., 2013). Another study showed that TUDCA decreased UPR signaling through all three branches of the UPR (IRE1, PERK, and ATF6) during exposure to tunicamycin (Berger and Haller, 2011). A study on type 1 diabetes, however, found the opposite; TUDCA appeared to improve cell survival through an increase in ATF6 levels in pancreatic β cells (Engin et al., 2013). It is worth noting that diabetic pancreatic β cells had a decreased baseline level of UPR signaling (lowered expression of XBP1 and ATF6 compared to control), suggesting that although these results differed from others in the literature, the net effect was a restoration of UPR signaling to "normal" levels in all cases. It has also been previously suggested that TUDCA may also reduce apoptosis by decreasing calcium release from intracellular compartments (including the ER) during exposure to the ER stressor thapsigargin, thereby reducing calcium-dependent caspase 12 activation (Xie et al., 2002).

Taken together, these data suggest that TUDCA may reduce cellular sensitivity to ER stress and reduce cell death. We aimed to study this using *Saccharomyces cerevisiae*, or budding yeast. Previous studies on chemical chaperones (such as 4-PBA, DMSO, TMAO, and glycerol) have utilized yeast models to assess the impact of these drugs on protein folding and trafficking, as well as downstream effects on conserved PQC machinery and pathways (Mai et al., 2018; Singh et al., 2007). The UPR is also highly conserved in yeast, but is orchestrated through a single stress sensing branch (Ire1/Hac1), making it much simpler to study and manipulate (Ron and Walter, 2007; Walter and Ron, 2011). By using yeast genetics, we can more easily characterize the impact of a variety of genes on TUDCA's ability to resolve ER stress or protein misfolding, allowing an in-depth assessment of TUDCA's mechanism of action. Given the connection between protein misfolding diseases and those improved by TUDCA, we propose that TUDCA may decrease misfolded protein burden, thereby lessening sensitivity to ER stress.

4.2 Results

4.2.1 TUDCA rescues Tm-induced ER stress, but rescue can be uncoupled from the UPR

First, to identify whether TUDCA treatment would be sufficient to rescue UPR deletion mutants exposed to ER stress, wild-type, *ire1*Δ and *hac1*Δ cells were spotted on agar plates containing tunicamycin (Tm) and with or without TUDCA (**Figure 4.1A, B**). A range of concentrations of TUDCA were assessed for their ability to rescue Tm-induced growth defects of wild-type cells, and 5 mM was identified as the most effective (**Supplemental Figure S4.1A**). All further experiments were therefore conducted using 5 mM TUDCA. TUDCA was shown to rescue wild-type growth defects at high Tm concentrations (**Figure 4.1A**). As expected, cells without functional UPR (*ire1*Δ and *hac1*Δ) were especially sensitive to ER stress and were therefore unable to grow on Tm-only plates, even at low concentrations. Addition of TUDCA, however, was able to rescue this growth defect at low Tm concentrations (**Figure 4.1B**). To identify whether TUDCA would exert a time-dependent rescue or be more protective against ER stress when added before Tm, cells were pre-treated with TUDCA before spotting on plates containing 2.5 µg/mL Tm +/- TUDCA (**Supplemental Figure S4.1B**). No differences in growth were observed between pre-treated cells and those exposed to Tm and TUDCA at the same time. Additionally, cells were treated with a range of concentrations of DTT, a reducing agent which leads to ER and oxidative stress, and assessed for growth defects in liquid culture when co-treated with TUDCA (**Supplemental Figure S4.2**). While DTT caused significant growth defects in a concentration-dependent manner, TUDCA was unable to rescue these growth defects even at relatively low concentrations, suggesting that TUDCA's effect could be limited to negating the consequences of Tm treatment.

Next, we assessed TUDCA's ability to reduce ER stress. One consequence of acute ER stress induced by Tm is the retrotranslocation of small ER proteins (such as Cpr5) from the ER to the cytoplasm, a phenomenon termed ER reflux (Igbaria et al., 2019; Snapp and Lajoie, 2019). Reflux can be observed in living cells by assessing the localization of an ER-localized GFP reporter, which is localized to the ER during normal conditions but redistributes to the cytosol during misfolded protein stress (Snapp and Lajoie, 2019).

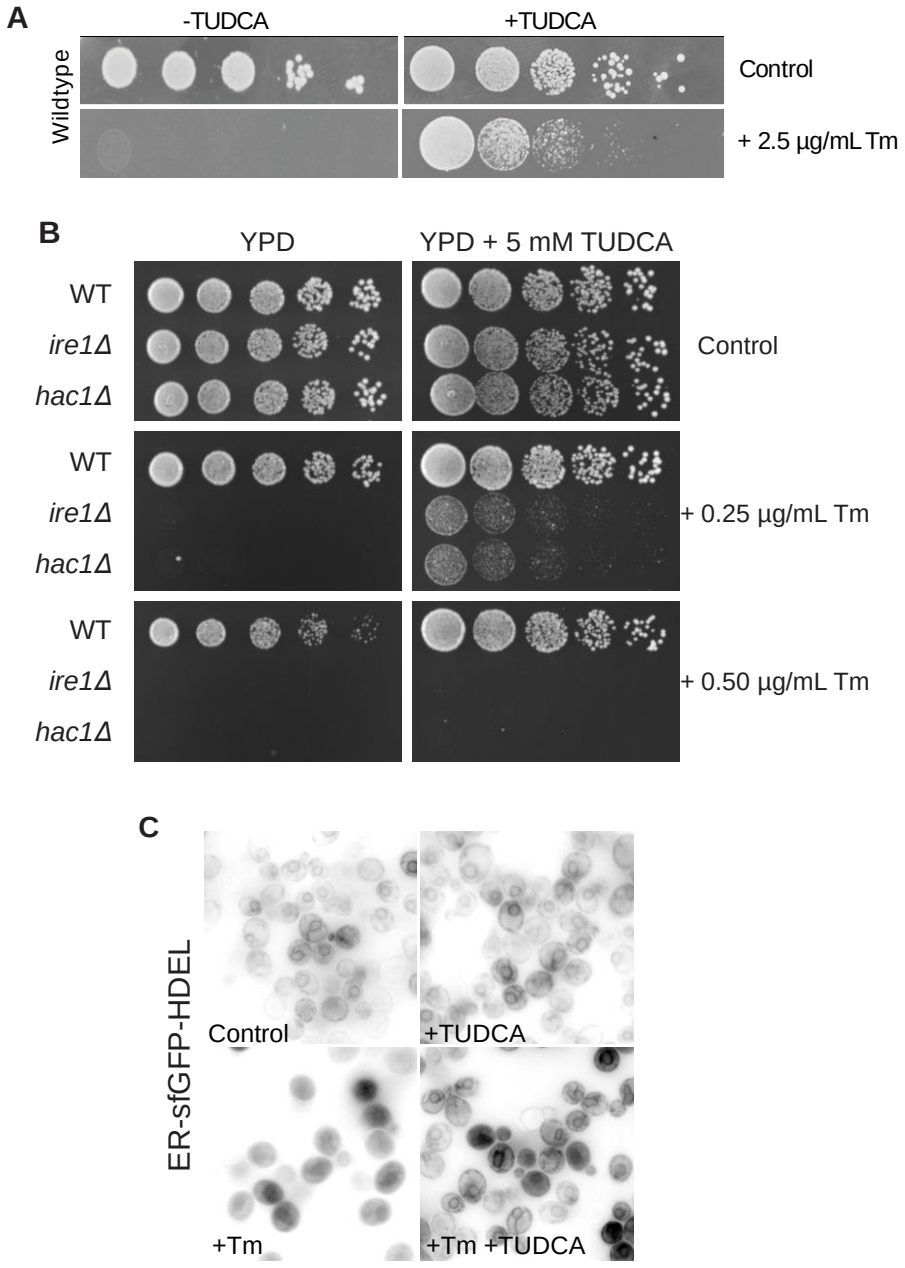

Figure 4.1: TUDCA increases resistance to ER stress independently of the UPR. A) Wild-type cells were spotted on YPD plates containing 0.25 µg/mL Tm, with or without TUDCA. **B)** Wild-type cells and those harbouring deletions in UPR effectors (*ire1*Δ and *hac1*Δ) were spotted on YPD plates containing 0.25-0.5 µg/mL Tm, with or without TUDCA. **C)** Cells expressing the fluorescent reporter ER-sfGFP-HDEL were treated with Tm, TUDCA, or both, and assessed qualitatively for fluorescence localization.

Cells treated with Tm showed this redistribution of fluorescence to the cytosol, but co-treatment with TUDCA reduced this (**Figure 4.1C**). To better understand why TUDCA was able to rescue ER stress, even in cells lacking UPR signaling (*ire1*Δ and *hac1*Δ), we next aimed to establish how TUDCA impacts UPR activation in wild-type yeast. Cells expressing the fluorescent UPR reporter, UPR-mCherry (Merksamer et al., 2008), were treated with a range of concentrations of Tm with or without TUDCA and assessed for UPR-mCherry expression over time (0-4 hours) by flow cytometry (**Figure 4.2A**). Interestingly, it was found that UPR-mCherry expression was increased with higher Tm concentrations and longer treatment times, as expected, but cells co-treated with 5 mM TUDCA had decreased UPR signaling compared to their controls in all cases. This is demonstrated by quantitation of mean fluorescent intensity value. To assess whether TUDCA could be decreasing protein expression overall (and not specifically UPR-related protein expression), cells expressing a different red fluorescent reporter protein under a constitutive promoter were also measured (**Figure 4.2B**). Median fluorescent intensity for these cells was consistent regardless of treatment or treatment time, indicating that TUDCA did not alter overall protein expression.

In addition to this, Tm's effect on the early stages of UPR activation was measured by *HAC1* splicing over time, as this splicing step occurs upstream of the transcription of UPR-related genes (including UPR-mCherry). Cells were treated with TUDCA, Tm, or both, and % splicing was quantified (**Figure 4.3**). *HAC1* splicing was assessed using 1 µg/mL Tm, which is a sub-lethal dose for wild-type cells with functional UPR. At this dose, cells are generally able to activate the UPR, resolve Tm-induced ER stress and misfolded proteins, and then attenuate UPR signaling. This attenuation step is required to prevent cell death from prolonged UPR activation (Chawla et al., 2011). These data show that, while Tm alone as well as Tm+TUDCA both increased *HAC1* splicing (demonstrating a similar initial activation of UPR), cells co-treated with TUDCA attenuated splicing more quickly than with Tm alone. These data could indicate that TUDCA improves the efficiency of the UPR, allowing it to resolve the same ER stress more quickly, or assists in resolving misfolded protein burden through other mechanisms. Similarly, we demonstrated that TUDCA could rescue defects in UPR attenuation using an Ire1-mutant strain, D828A, which contains a point mutation in its kinase domain

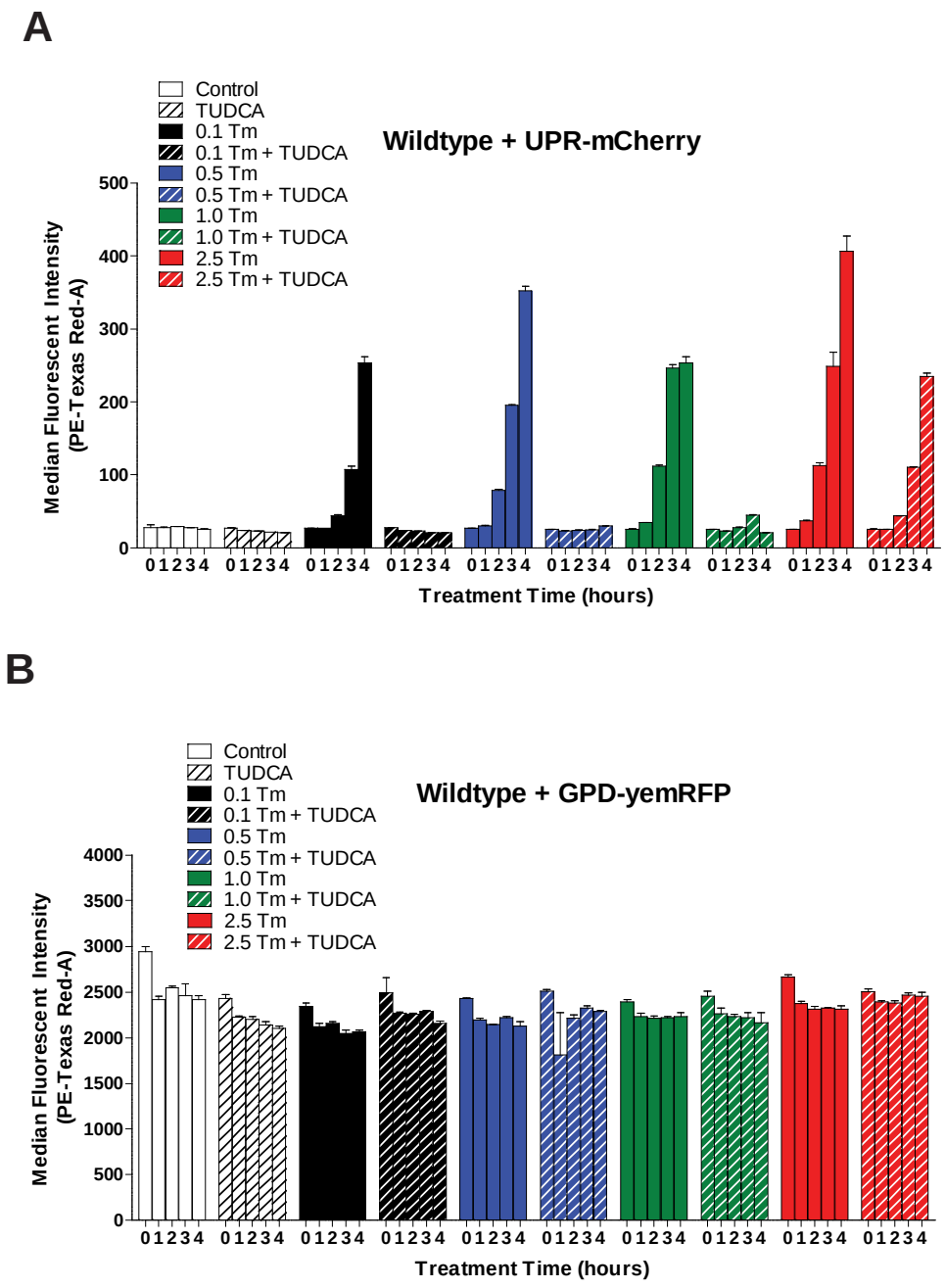

Figure 4.2: TUDCA decreases UPR signaling during Tm-induced ER stress. A) BY4742 cells expressing UPR-mCherry were grown to mid-log phase and treated with a range of Tm concentrations (in µg/mL) +/- 5 mM TUDCA for the indicated time periods. The same experiment was conducted with BY4742 + GPD-yemRFP (**B**) cells as well. UPR-mCherry/yem-RFP expression was assessed by flow cytometry, and median fluorescent intensity values of the cells in each sample +/- SEM were determined for each condition (n=3).

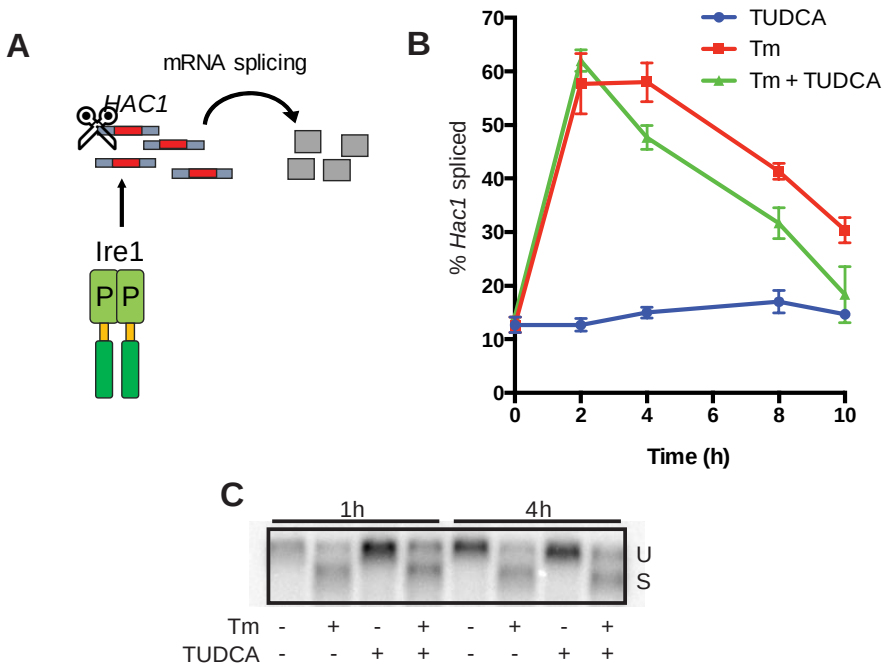

Figure 4.3: TUDCA causes faster attenuation of UPR signaling after ER stress. Wild-type cells were treated with the indicated combinations of Tm and TUDCA and assessed for UPR activated splicing of *HAC1* mRNA by Ire1 (**A**). Total RNA was isolated from cells at the indicated timepoints post-treatment, separated by formaldehyde gel electrophoresis, transferred to membranes, and assessed by northern blot. The ratio of spliced (lower molecular weight) vs. unspliced (higher molecular weight) species was calculated for three independent experiments (n=3) (**B**). A representative image of *HAC1* splicing is shown (**C**).

(Chawla et al., 2011). Cells expressing this mutant form of Ire1 are hypersensitive to ER stressors (including Tm) due to an inability to attenuate UPR signaling. These cells showed a concentration-dependent growth defect on plates containing Tm, but this defect was rescued by TUDCA at all but the highest Tm dosage (1 µg/mL) (**Supplemental Figure S4.3**).

Finally, we aimed to determine whether TUDCA was altering UPR signaling overall or UPR signaling in response to Tm-induced stress specifically. To this end, we used gene deletion strains with high baseline levels of UPR activation and treated them with TUDCA, then measured changes in UPR-mCherry by flow cytometry. *spf1*Δ cells (lacking an ER ion transporter/ATPase, which causes constitutive UPR activation) (Cronin et al., 2002), *scj1*Δ cells (lacking an ER chaperone protein, also causes constitutive UPR activation) (Silberstein et al., 1998), and *ccw12*Δ cells (lacking a cell wall mannoprotein, causes sensitivity to cell wall and osmotic stress) were treated with TUDCA for up to 24 hours (**Figure 4.4**). These cells showed no change in UPR-mCherry expression with the addition of TUDCA, indicating that TUDCA was not able to rescue UPR activation caused by genetic modifications. This suggests that it directly alters the cellular effects of Tm as opposed to lowering UPR expression in general.

4.2.2 Tm's effect on protein glycosylation and UPR activation is reduced by co-treatment with TUDCA

To begin to identify exactly how TUDCA was rescuing Tm-induced growth defects, differences in glycosylation with Tm +/- TUDCA were assessed (**Figure 4.5A**). Since Tm causes ER stress by blocking N-linked glycosylation (leading to a buildup of immature glycoproteins in the ER) (Kuo and Lampen, 1974), we examined changes in the proportion of glycosylation species of PDI1 (protein disulfide isomerase 1). Loss of glycosylation is indicated by an increase in the amount of precursor species (lower molecular weight bands) and a decrease in the fully-glycosylated species (higher molecular weight bands). Cells were grown to mid-log phase then treated with a range of Tm concentrations (including very low doses to ensure that cells survived the treatment), with or without TUDCA, for two hours. Protein was extracted and PDI1 expression was assessed by western blot. It was found that TUDCA was able to restore glycosylation of

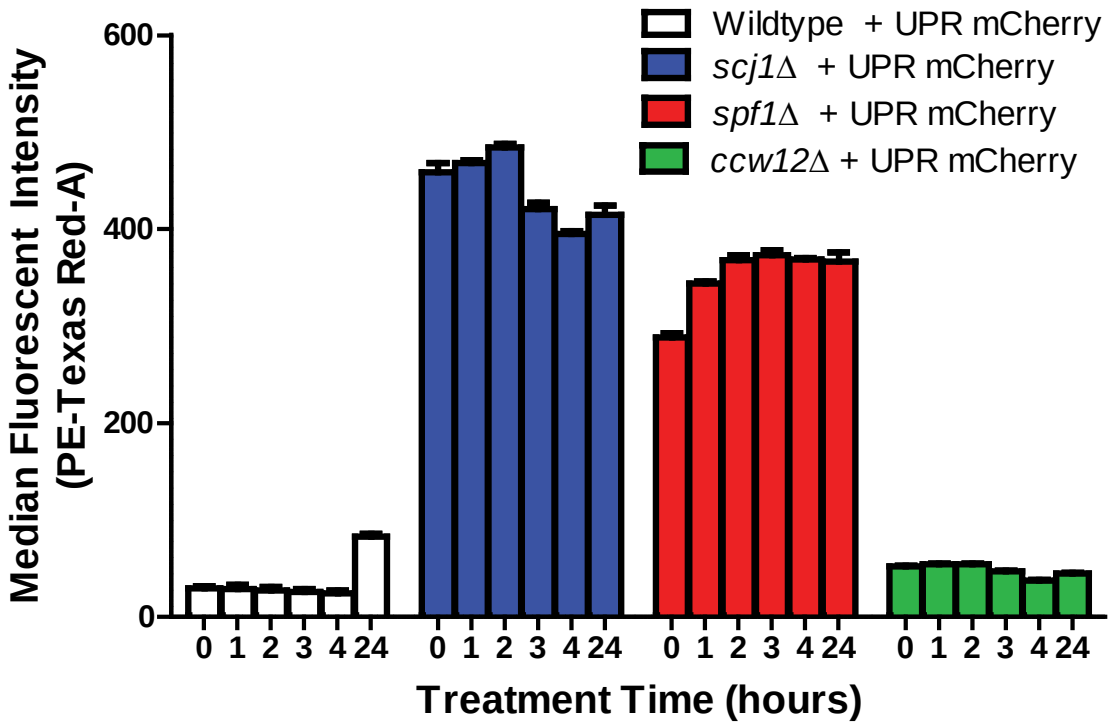

Figure 4.4: TUDCA is unable to compensate for UPR caused by ER defects. Wild-type cells and those harbouring deletions in ER- and cell wall-related genes expressing UPR-mCherry were grown to mid-log phase and treated with a range of Tm concentrations (in µg/mL) +/- 5 mM TUDCA for the indicated time periods. UPR-mCherry expression was assessed by flow cytometry, and median fluorescent intensity values were determined for each condition +/-SEM (n=3).

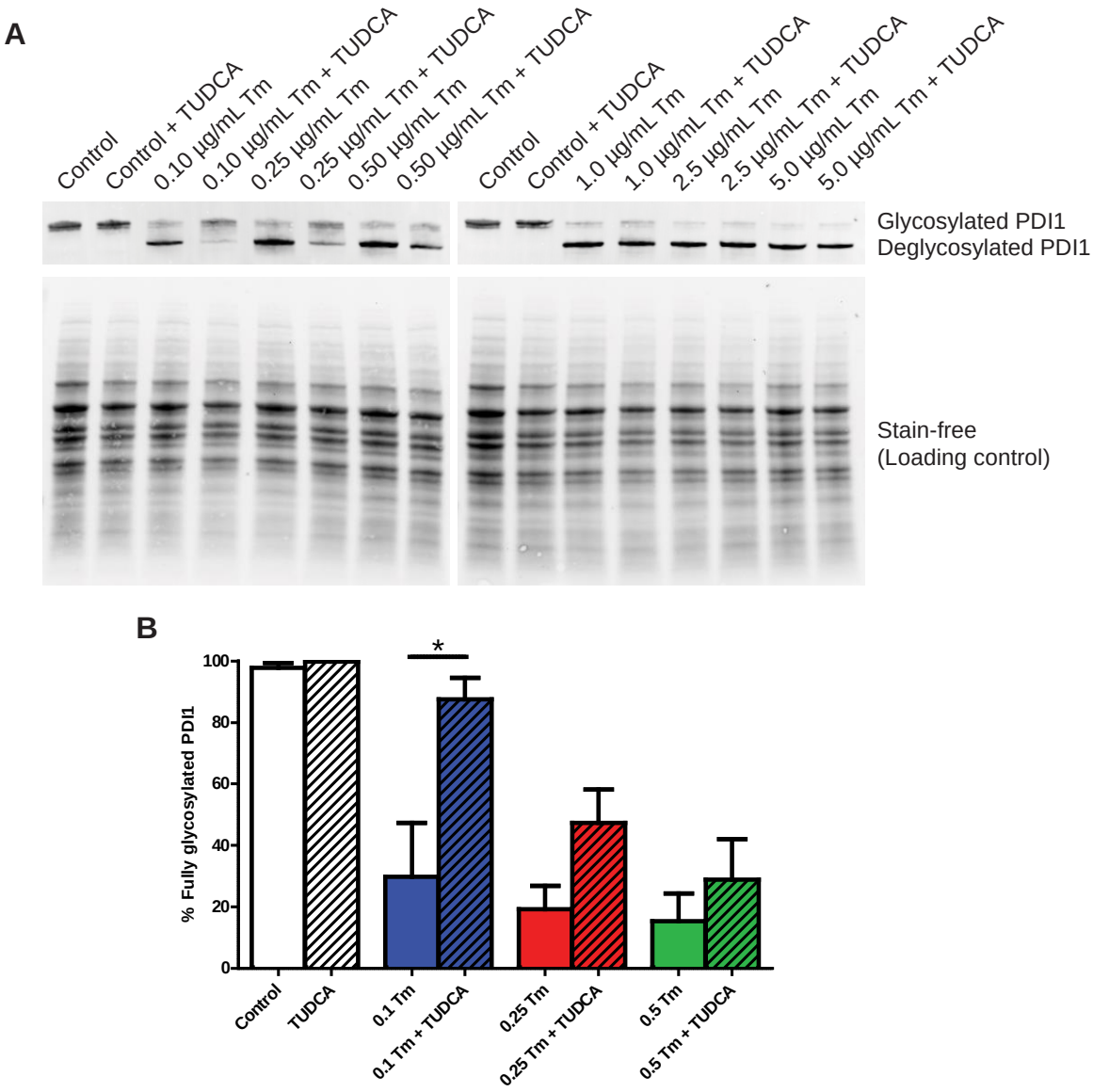

Figure 4.5: TUDCA rescues N-linked glycosylation defect caused by low concentrations of Tm. A) Wild-type cells were grown to mid-log phase (around 3h, OD_{600} 0.5) then treated for 2h with the indicated concentrations of Tm and TUDCA. Protein was isolated and assessed by western blot for PDI1 and stain-free signal (control). Deglycosylated PDI1 is seen in the lower molecular weight bands. **B)** Densitometric analysis was performed for lower Tm concentrations and normalized using stain-free images. Percentage of glycosylated PDI1 (upper bands) vs. deglycosylated PDI1 (lower bands) was calculated for each condition and represented +/- SEM. Statistical analysis was performed using t-tests (n=3, $P = 0.0377$) .

PDI1 at the low Tm concentrations (0.1-0.5 µg/mL), but not at the higher Tm concentrations (1.0-5.0 µg/mL) (**Figure 4.5B**). These results suggest that TUDCA compensates for the defects in glycosylation caused by Tm, possibly either by negating its effects or blocking its entry into the cell, but that there is a limit to the amount of Tm it is able to block. These results match our spot assay data from **Figure 4.1** wherein TUDCA rescues Tm-induced growth defects of UPR deletion mutants, but only at low concentrations. We also demonstrated a similar concentration-dependent effect with the hyperactive Ire1 D828A mutant, which is unable to attenuate the UPR, as shown in **Supplemental Figure S4.3**.

Since our work in Chapter 3 identified a role for stress tolerance in chronological aging, and since lithocholic acid (another bile acid) has been shown to extend yeast chronological lifespan (Burstein et al., 2012; Goldberg et al., 2010), we tested whether TUDCA could increase longevity by reducing this form of stress as well (**Supplemental Figure S4.4**). We found that TUDCA modestly increased survival integral/area under the curve of wild-type cells, although the aging endpoint remained unchanged.

4.2.3 The cell wall integrity pathway is involved in TUDCA's mechanism of action

Having demonstrated that TUDCA rescues Tm-induced ER stress, but appeared to do so independently of the UPR, we aimed to understand which signaling pathways were involved in its mechanism. Using a gene deletion library screen (**Figure 4.6A, B**), we identified genes which, when deleted, prevent the rescue of Tm-induced growth defects by TUDCA. Gene deletion strains not rescued by TUDCA could indicate the genes likely involved in TUDCA's mechanism of action. Several of the genes identified with this screen were UPR-related (*IRE1, HAC1*), but several were related to other stress responses, including *SLT2* and *BCK1* which are involved with the cell wall integrity pathway and response to cell wall stress (**Table 4.1**). In fact, when analyzed by functional properties using TheCellMap, which identifies genetic interactions based on synthetic gene arrays and then clusters related genes by gene ontology terms (Usaj et al., 2017), a large cluster of the genes not rescued by TUDCA were shown to be involved in glycosylation, protein folding, and the cell wall (**Figure 4.6C**). We hypothesized that

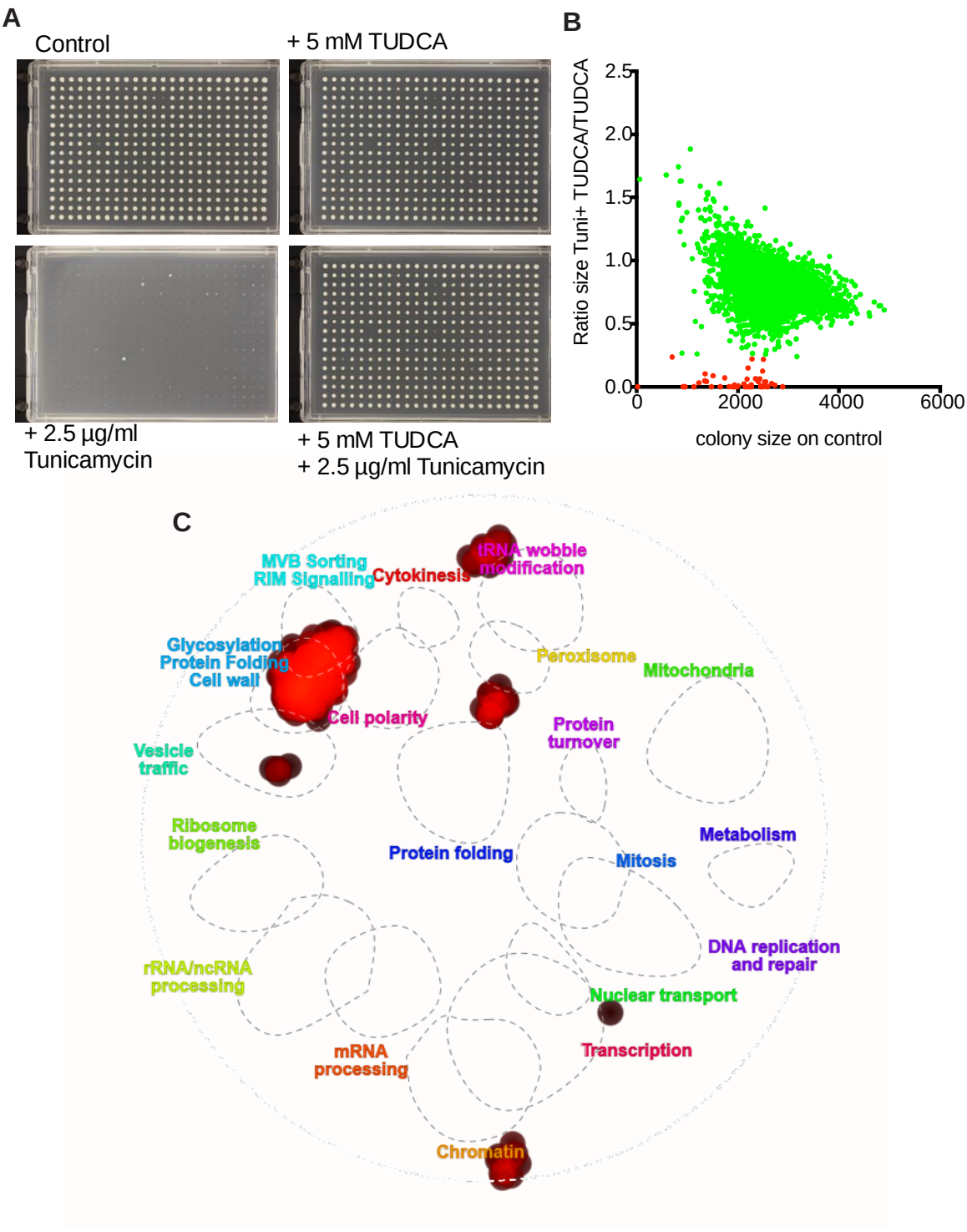

Figure 4.6: Deletions in UPR or related stress response pathways prevent TUDCA's rescue of ER stress, suggesting involvement in its mechanism of action. A BY4742 gene deletion library was spotted on plates containing the indicated concentrations of Tm and TUDCA (**A**). Colony size on the control plate was quantified and plotted against the

ratio of colony sizes on the Tm plate vs. Tm + TUDCA to determine whether growth was rescued by addition of TUDCA (**B**). A colony size ratio of <0.25 was considered “Not rescued”, and gene deletions in this category were identified and classified using TheCellMap (Usaj et al., 2017) (**C**).

Table 4.1: Description of gene deletions not rescued by TUDCA

Name	ORF	Colony Size Ratio: Tm + TUDCA/ TUDCA	Description
ACK1	YDL203C	0.003384913	Protein that functions upstream of Pkc1p in the cell wall integrity pathway; GFP-fusion protein expression is induced in response to the DNA-damaging agent MMS; non-tagged Ack1p is detected in purified mitochondria
ARO1	YDR127W	0.049960349	Pentafunctional arom protein, catalyzes steps 2 through 6 in the biosynthesis of chorismate, which is a precursor to aromatic amino acids
BCK1	YJL095W	0	Mitogen-activated protein (MAP) kinase kinase kinase acting in the protein kinase C signaling pathway, which controls cell wall integrity; upon activation by Pkc1p phosphorylates downstream kinases Mkk1p and Mkk2p
BEM2	YER155C	0.084076433	Rho GTPase activating protein (RhoGAP) involved in the control of cytoskeleton organization and cellular morphogenesis; required for bud emergence
BTS1	YPL069C	0	Geranylgeranyl diphosphate synthase, increases the intracellular pool of geranylgeranyl diphosphate, suppressor of bet2 mutation that causes defective geranylgeranylation of small GTP-binding proteins that mediate vesicular traffic

BUD19	YJL188C	0.196190476	Dubious open reading frame, unlikely to encode a protein; not conserved in closely related Saccharomyces species; 88% of ORF overlaps the verified gene RPL39; diploid mutant displays a weak budding pattern phenotype in a systematic assay
CNB1	YKL190W	0	Calcineurin B; the regulatory subunit of calcineurin, a Ca^{2+}/calmodulin-regulated type 2B protein phosphatase which regulates Crz1p (a stress-response transcription factor), the other calcineurin subunit is encoded by CNA1 and/or CMP1
CTK1	YKL139W	0	Catalytic (alpha) subunit of C-terminal domain kinase I (CTDK-I), which phosphorylates both RNA pol II subunit Rpo21p to affect transcription and pre-mRNA 3' end processing, and ribosomal protein Rps2p to increase translational fidelity
ECM31	YBR176W	0	Ketopantoate hydroxymethyltransferase, required for pantothenic acid biosynthesis, converts 2-oxoisovalerate into 2-dehydropantoate
ENV9	YOR246C	0.078108941	Protein with similarity to oxidoreductases, found in lipid particles; required for replication of Brome mosaic virus in *S. cerevisiae*, a model system for studying replication of positive-strand RNA viruses in their natural hosts
FRE8	YLR047C	0	Protein with sequence similarity to iron/copper reductases, involved in iron

			homeostasis; deletion mutant has iron deficiency/accumulation growth defects; expression increased in the absence of copper-responsive transcription factor Mac1p
HAC1	YFL031W	0	Basic leucine zipper (bZIP) transcription factor (ATF/CREB1 homolog) that regulates the unfolded protein response, via UPRE binding, and membrane biogenesis; ER stress-induced splicing pathway facilitates efficient Hac1p synthesis
HOG1	YLR113W	0.018774704	Mitogen-activated protein kinase involved in osmoregulation via three independent osmosensors; mediates the recruitment and activation of RNA Pol II at Hot1p-dependent promoters; localization regulated by Ptp2p and Ptp3p
INP52	YNL106C	0	Polyphosphatidylinositol phosphatase, dephosphorylates a number of phosphatidylinositols (PIs) to PI; involved in endocytosis; hyperosmotic stress causes translocation to actin patches; synaptojanin-like protein with a Sac1 domain
IRE1	YHR079C	0	Serine-threonine kinase and endoribonuclease; transmembrane protein that mediates the unfolded protein response (UPR) by regulating Hac1p synthesis through *HAC1* mRNA splicing; Kar2p binds inactive Ire1p and releases from it upon ER stress

MDM31	YHR194W	0	Mitochondrial inner membrane protein with similarity to Mdm32p, required for normal mitochondrial morphology and inheritance; interacts genetically with *MMM1*, *MDM10*, *MDM12*, and *MDM34*
MNN10	YDR245W	0.012057272	Subunit of a Golgi mannosyltransferase complex also containing Anp1p, Mnn9p, Mnn11p, and Hoc1p that mediates elongation of the polysaccharide mannan backbone; membrane protein of the mannosyltransferase family
MNN11	YJL183W	0.010191083	Subunit of a Golgi mannosyltransferase complex that also contains Anp1p, Mnn9p, Mnn10p, and Hoc1p, and mediates elongation of the polysaccharide mannan backbone; has homology to Mnn10p
NBP2	YDR162C	0	Protein involved in the HOG (high osmolarity glycerol) pathway, negatively regulates Hog1p by recruitment of phosphatase Ptc1p the Pbs2p-Hog1p complex, found in the nucleus and cytoplasm, contains an SH3 domain that binds Pbs2p
NHA1	YLR138W	0	Na^+/H^+ antiporter involved in sodium and potassium efflux through the plasma membrane; required for alkali cation tolerance at acidic pH
PHO80	YOL001W	0	Cyclin, negatively regulates phosphate metabolism; Pho80p-Pho85p (cyclin-CDK complex) phosphorylates Pho4p and Swi5p; deletion of *PHO80* leads to aminoglycoside

			supersensitivity; truncated form of *PHO80* affects vacuole inheritance
PTC1	YDL006W	0	Type 2C protein phosphatase (PP2C); inactivates the osmosensing MAPK cascade by dephosphorylating Hog1p; mutation delays mitochondrial inheritance; deletion reveals defects in precursor tRNA splicing, sporulation and cell separation
RGD1	YBR260C	0.178941626	GTPase-activating protein (RhoGAP) for Rho3p and Rho4p, possibly involved in control of actin cytoskeleton organization
RLM1	YPL089C	0.051295074	MADS-box transcription factor, component of the protein kinase C-mediated MAP kinase pathway involved in the maintenance of cell integrity; phosphorylated and activated by the MAP-kinase Slt2p
RMD7	YER083C	0	Subunit of the GET complex; involved in insertion of proteins into the ER membrane; required for the retrieval of HDEL proteins from the Golgi to the ER in an ERD2 dependent fashion and for meiotic nuclear division
SLT2	YHR030C	0	Serine/threonine MAP kinase involved in regulating the maintenance of cell wall integrity and progression through the cell cycle; regulated by the PKC1-mediated signaling pathway
SNF5	YBR289W	0	Subunit of the SWI/SNF chromatin remodeling complex involved in transcriptional regulation; functions

			interdependently in transcriptional activation with Snf2p and Snf6p
SNF6	YHL025W	0.109725686	Subunit of the SWI/SNF chromatin remodeling complex involved in transcriptional regulation; functions interdependently in transcriptional activation with Snf2p and Snf5p
SPF1	YEL031W	0.024152107	P-type ATPase, ion transporter of the ER membrane involved in ER function and Ca^{2+} homeostasis; required for regulating Hmg2p degradation; confers sensitivity to a killer toxin (SMKT) produced by Pichia farinosa KK1
SSK1	YLR006C	0.07628866	Cytoplasmic response regulator, part of a two-component signal transducer that mediates osmosensing via a phosphorelay mechanism; dephosphorylated form is degraded by the ubiquitin-proteasome system; potential Cdc28p substrate
SSP2	YOR242C	0.046464646	Sporulation specific protein that localizes to the spore wall; required for sporulation at a point after meiosis II and during spore wall formation; SSP2 expression is induced midway in meiosis
SWE1	YJL187C	0.062539482	Protein kinase that regulates the G2/M transition by inhibition of Cdc28p kinase activity; localizes to the nucleus and to the daughter side of the mother-bud neck; homolog of *S. pombe* Wee1p; potential Cdc28p substrate

TPO1	YLL028W	0.023743017	Polyamine transporter that recognizes spermine, putrescine, and spermidine; catalyzes uptake of polyamines at alkaline pH and excretion at acidic pH; phosphorylation enhances activity and sorting to the plasma membrane
VPS24	YKL041W	0.000543183	One of four subunits of the endosomal sorting complex required for transport III (ESCRT-III); forms an ESCRT-III subcomplex with Did4p; involved in the sorting of transmembrane proteins into the multivesicular body (MVB) pathway

TUDCA could potentially block Tm's entry into the cell via the cell wall integrity (CWI) pathway. CWI and UPR signaling have previously been linked; it has been shown that cell wall stress activates UPR-related genes, and ER stress can activate CWI signaling (Krysan, 2009; Scrimale et al., 2009). CWI, then, could feasibly play a role in ER stress tolerance. We found that cells with a deletion in a key CWI gene, *SLT2*, are unable to grow in the presence of TUDCA alone. However, when supplemented with the osmotic stabilizer sorbitol (which can rescue cell wall defects), growth was restored (**Figure 4.7A**). *slt2*Δ cells have also been shown to be hypersensitive to Tm/ER stress, and Slt2 is upregulated during Tm exposure (Chen et al., 2005). This could further indicate that TUDCA impacts Slt2/CWI signaling in response to Tm. Another example of this potential connection between CWI and UPR can be found in cells expressing Bck1-20, a hyperactive mutant of the Bck1 protein (Lee and Levin, 1992). As mentioned, Bck1 is involved with the yeast cell wall integrity pathway; when deleted, cells become hypersensitive to ER and osmotic stress and will lyse unless supported by an osmotic stabilizer such as sorbitol (Lee and Levin, 1992). When hyperactive, however, this protein conveys a higher tolerance to stressors, including ER stressors like Tm, as demonstrated by our own lab and others (Ahmed et al., 2019; Chen et al., 2005). In accordance with this, we found that cells expressing Bck1-20 showed decreased UPR-mCherry expression upon treatment with Tm (**Supplemental Figure S4.5**). This offers an interesting example of a link between increased cell wall integrity signaling and increased tolerance to ER stress.

The CWI pathway has also been shown to interact with calcium/calcineurin-dependent signaling pathways. Calcineurin is a protein phosphatase required for many Ca^{2+}-dependent signaling pathways which has also been identified as an essential protein for cellular growth in high osmotic stress environments (Garrett-Engele et al., 1995). Cells with defective CWI genes (including deletions in *PKC1* or *SLT2/MPK1*) exhibit a more severe growth defect when lacking functional calcineurin, and a less severe growth defect when calcineurin is constitutively active (Garrett-Engele et al., 1995). Calcium signaling pathways are also linked to ER stress and the UPR; calcium influx from the plasma membrane and activation of calcineurin have both been shown to occur when misfolded

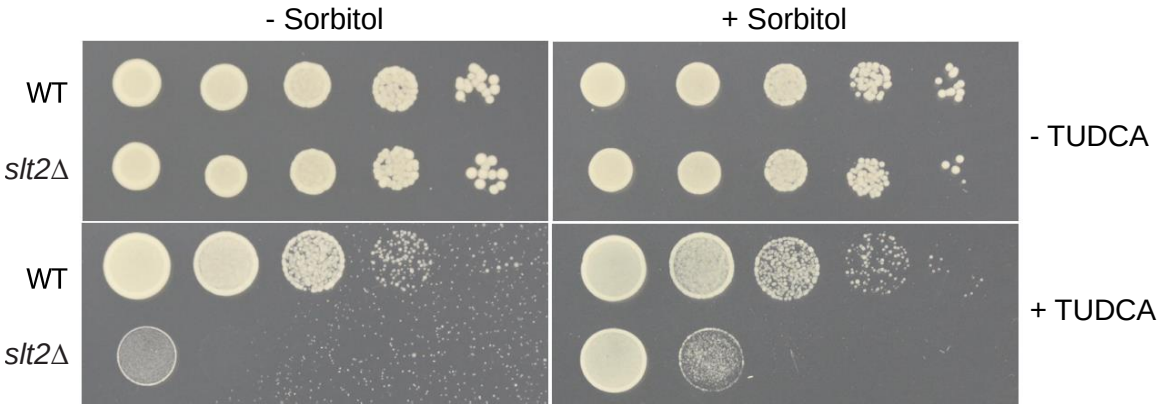

Figure 4.7: Cell wall integrity deletion confers sensitivity to TUDCA. Wild-type and *slt2*Δ cells were diluted to OD_{600}=0.2 and spotted in serial fivefold dilutions on SC plates, with or without the osmotic stabilizer Sorbitol and with or without TUDCA. Plates were incubated at 30°C for two days before imaging.

proteins accumulate in the ER, and loss of calcineurin genes causes enhanced sensitivity to Tm (Bonilla et al., 2002). As a result, we briefly aimed to identify whether Ca^{2+} signaling was involved in TUDCA's mechanism. We first did so using BAPTA, a Ca^{2+}-chelator; when added to the media, it would theoretically block any calcium-mediated rescue of Tm sensitivity by TUDCA if this were the mechanism of action. Wild-type, UPR deletion mutant, and calcineurin deletion mutant (*cnb1*Δ) cells were spotted on plates containing combinations of Tm, TUDCA, and BAPTA (**Supplemental Figure S4.6A**). BAPTA did not negate or alter TUDCA's rescue of Tm-induced stress in any of these strains. Additionally, when treated with lower concentrations of Tm to allow deletion strains to grow, *cnb1*Δ cells were rescued by the addition of TUDCA much like UPR deletion mutants (**Supplemental Figure S4.6B**); if calcineurin signaling were required for TUDCA's rescue, no growth would have been expected. This suggests that TUDCA's impact on CWI signaling is not occurring indirectly through calcium-related pathways.

4.2.4 TUDCA rescues cell wall stress and improves cell wall integrity

To test our hypothesis that TUDCA may exert its effects via CWI signaling, we assessed whether TUDCA could rescue cell wall stress in addition to ER stress. We spotted wild-type cells on plates containing calcofluor white (CFW), a cell wall-specific stressor, with or without TUDCA. We also spotted wild-type cells pretreated with TUDCA overnight to assess whether TUDCA exerted a time-dependent effect on the cell wall (**Supplemental Figure S4.1C**). Cells grown on CFW plates displayed a severe growth defect, regardless of TUDCA pretreatment, compared to the control. When TUDCA is added to CFW plates, however, this growth defect is rescued. This confirms that TUDCA is able to rescue cell wall-specific stress, but that pretreatment with TUDCA was not important for this rescue. Similar to the rationale presented in **Figure 4.1**, we next wanted to see whether this rescue of cell wall stress was still possible when UPR components were deleted. To this end, wild-type and *ire1*Δ cells were spotted on plates containing a range of CFW or caspofungin (another cell wall stressor) concentrations, with or without TUDCA (**Figure 4.8**). TUDCA improved growth of both cell types across all CFW

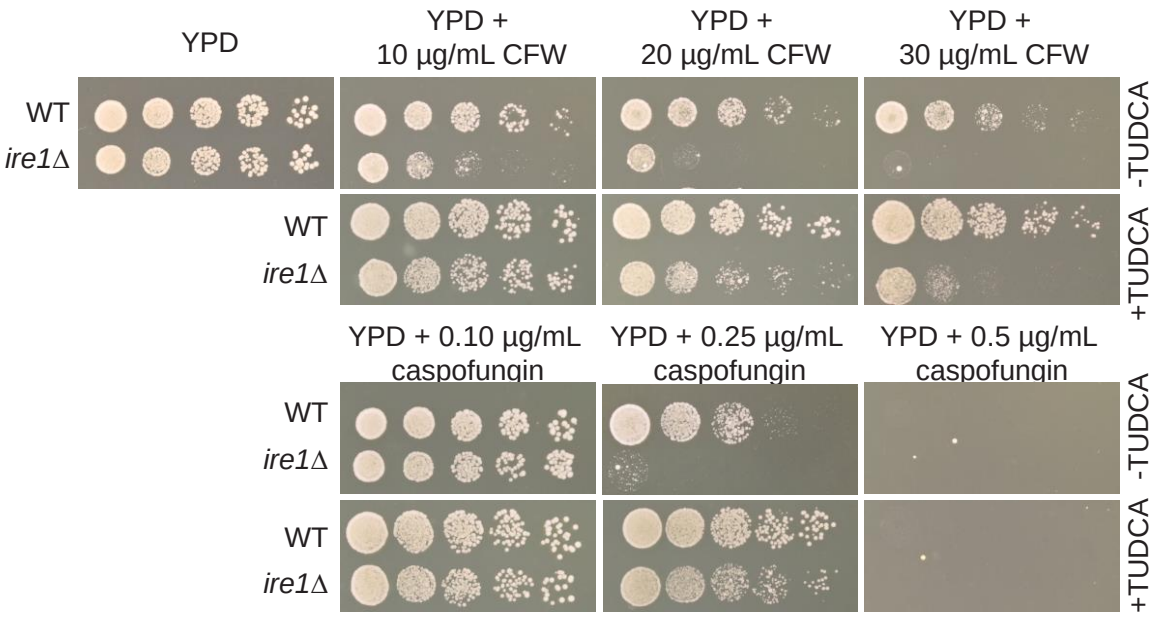

Figure 4.8: TUDCA rescues cell wall stress independently of the UPR. Wild-type or *ire1*Δ cells were spotted in serial fivefold dilutions on YPD plates with or without various concentrations of the cell wall stressors calcofluor white (CFW) and caspofungin, with or without TUDCA. Plates were incubated at 30°C for two days before imaging.

concentrations; wild-type cells showed a complete rescue, while *ire1*Δ cells showed complete rescue at 10 µg/mL CFW and a partial rescue at 20 and 30 µg/mL. TUDCA was also able to rescue growth of both cell types at 0.25 µg/mL caspofungin. Since TUDCA was able to restore growth of a UPR-deficient mutant in response to cell wall stress, these data provide further evidence that, although TUDCA rescues ER stress and improves UPR efficiency, it is unlikely that it does so directly through UPR signaling.

Given that CWI stress can activate the UPR and vice versa, we propose that TUDCA acts by preventing or attenuating stress in the presence of stressors, likely through the CWI pathway (including Slt2) or other stress response pathways (e.g. Hog1/osmotic stress). This would explain how TUDCA is still able to rescue ER stress in cells without functional UPR signaling; by activating other stress response pathways, lack of UPR signaling could be circumvented in these instances. To directly measure whether the physical integrity of the cell wall was being affected by TUDCA, a fluorescence quenching experiment was performed (**Figure 4.9**). Cells were treated with a fluorescent dye, FM4-64, which is able to pass through the cell wall and stain the plasma membrane (and other cellular compartments with prolonged exposure). FM4-64 fluorescence can be quenched by a cell wall-impermeable dye, trypan blue. If the cell wall is intact, FM4-64 fluorescence is unaffected by extracellular trypan blue. If, however, the cell wall is compromised, fluorescent quenching occurs and the signal dims (**Figure 4.9A**). We found that cells grown to mid-log phase in media containing 5 mM TUDCA showed significantly reduced quenching compared to the control. As a positive control for quenching, cells were also treated with SDS to disrupt the cell wall. TUDCA treated cells showed relatively little loss of fluorescence, suggesting that TUDCA improves CWI (**Figure 4.9B-C**).

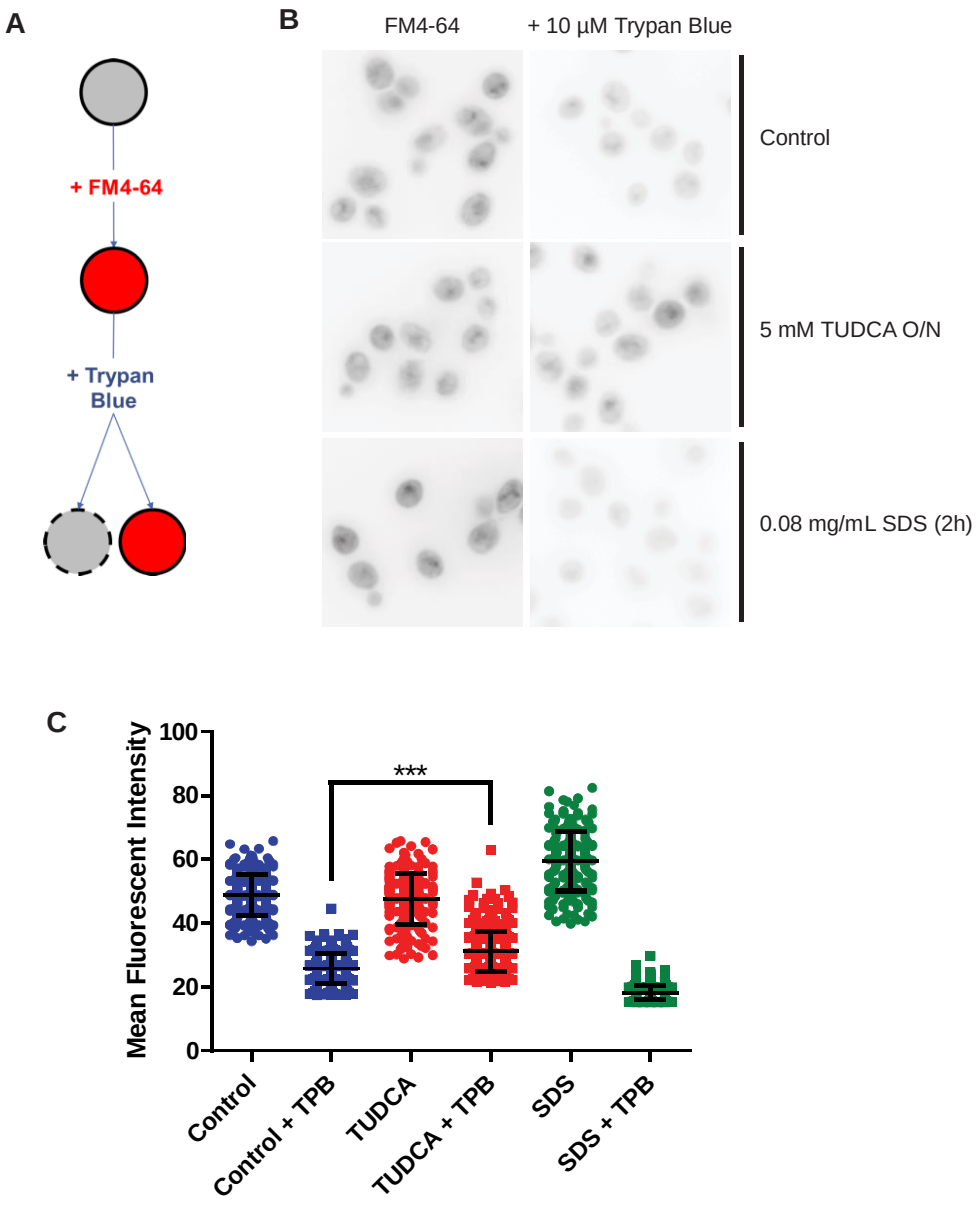

Figure 4.9: TUDCA reduces cell wall porosity. Wild-type cells were grown to mid-log phase (around 3h, OD_{600} 0.5) with or without 5 mM TUDCA before assessment for fluorescence quenching (**A**). **B)** Cells were treated with 50 µM FM4-64 dye for 3 minutes on ice. Cells were washed and imaged. For quenching, 10 µM trypan blue was added to each sample and then imaged immediately. As a positive control for disrupted cell wall integrity, cells were also treated with 0.08 mg/mL SDS two hours before FM4-64/trypan blue staining. Inverted images are shown for clarity. **C)** Fluorescent intensity for individual cells in each condition was measured using ImageJ and statistical analysis was performed using t-tests (n=203-412 individual cells, $P < 0.0001$).

4.3 Discussion

We have shown here that TUDCA plays an important role in the regulation of ER stress, but that this role can be uncoupled from the UPR. We have shown that, while TUDCA is able to reduce UPR signaling upon exposure to stress, it is also able to rescue stress when cells lack functional UPR, suggesting a mechanism that can circumvent UPR signaling. This is a particularly important finding, since very few mechanisms are currently known that can block the effect of the ER stress-inducing drug, Tm. Deletion or depletion of the transporter responsible for Tm uptake in mammalian cells, MFSD2A, is typically the only method to prevent Tm sensitivity (Bassik and Kampmann, 2011; Reiling et al., 2011). In this study, however, we have shown that TUDCA is able to directly block/negate the effects of Tm on N-linked glycosylation. We propose that this occurs through involvement of the cell wall integrity pathway (**Figure 4.10**). Our fluorescence quenching data support this; TUDCA improved physical cell wall integrity and reduced permeability compared to untreated cells. The fact that cells lacking a key CWI gene (*SLT2*) are unable to grow in the presence of TUDCA also supports this. It should be acknowledged, however, that Slt2 does play other roles in the cell: it is also involved in the ER stress surveillance pathway (ERSU) and can be activated by ER stressors independently of the UPR (Babour et al., 2010). Despite this, Slt2's involvement here is still more likely to be related to its role in CWI signaling, since addition of a cell wall stabilizer, sorbitol, rescues the growth defect seen with TUDCA when *SLT2* is deleted.

We have also shown that TUDCA can rescue multiple forms of stress, including treatment with ER and cell wall stressors. TUDCA's ability to rescue stress did not, however, extend to the oxidative and ER stress caused by DTT. We have previously shown that DTT and Tm can affect cells differently; in Chapter 3, *KAR2-upre*^{d} cells were hypersensitive to Tm, but unaffected by DTT (Chadwick et al., 2019). These differences could be explained by the fact that DTT has been previously shown to induce stress more quickly than Tm (possibly limiting the amount of time for TUDCA to exert its effects), and to induce the transcription of a different set ER stress-related genes (Li et al., 2011). TUDCA also modestly extended chronological lifespan in wild-type cells, which was shown in our previous study to require the UPR/normal stress tolerance, suggesting that

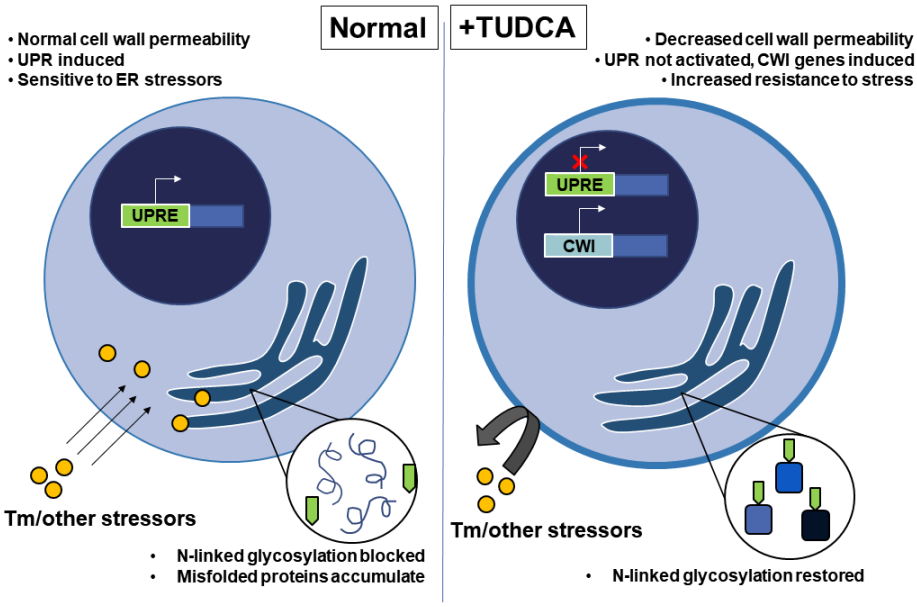

Figure 4.10: Model of TUDCA's mechanism of action. We propose that TUDCA modulates ER stress by activating CWI pathways, thereby preconditioning the cells for stress tolerance, and by decreasing cell wall porosity, limiting the entry of Tm or other stressors.

TUDCA improves these (Chadwick et al., 2019). It could not, however, reduce UPR signaling in genetic forms of stress caused by ER or cell wall component deletions (such as *SPF1, SCJ1,* and *CCW12*). Unexpectedly, pre-treating cells with TUDCA before stress exposure showed very little difference from concurrent stress/TUDCA treatment, suggesting that TUDCA may exert its effects relatively quickly.

As previously mentioned, CWI signaling is linked to the induction of several other stress response pathways including the osmotic, heat shock, and generalized stress responses (Arroyo et al., 2009; Boorsma et al., 2004). If TUDCA works by entering the cell and activating CWI pathways, it may thereby reduce sensitivity to other forms of stress via these signaling pathway intersections; it might "prime" the cells to respond more quickly or more efficiently to stressors. This is a mechanism previously described; preconditioning with a mild or moderate stress can often lead to a more efficient later response to severe stress (Hayakawa et al., 2009; Mollereau, 2015). Alternatively, it is possible that TUDCA works externally only; it may only improve cell wall assembly or change the composition of the cell wall from the outside, preventing the entry of chemical stressors. The aforementioned Tm transporter MFSD2A is not known to be expressed in yeast, but it should be noted that, even in mammalian cells with the MFSD2A transporter, some Tm can cross the plasma membrane through simple diffusion (Reiling et al., 2011). TUDCA may change this rate of diffusion by limiting the Tm that moves through the cell wall and reaches the plasma membrane, thereby limiting the Tm which can then wreak havoc on protein folding in the ER. TUDCA also rescues cell wall stressors, however, which can have different mechanisms of entry. Calcofluor white, for example, does not enter the cell at all, instead binding to the cell wall and disrupting chitin structure externally (Heilmann et al., 2013). Caspofungin, on the other hand, is a cell wall stressor with an internal target – caspofungin inhibits subunits of 1,3-β glucan synthase, preventing production of the cell wall component (Walker et al., 2015). Future studies into TUDCA's mechanism of action will likely be needed to decipher this, but it is possible that TUDCA's effect on the cell wall could both reduce the effect of external cell wall disruptions (by increasing cell wall thickness or structural integrity) and internally acting stressors (by preventing their uptake). Previous studies have shown that cell wall structure is an important determinant of sensitivity to stress (Lambert, 2002;

Mishra et al., 2007), and that deletion of CWI components sensitizes fungi to anti-fungal drugs (Gow et al., 2017; Islahudin et al., 2013). Examining TUDCA's impact on the biochemical and physical composition of the cell wall or on the activity of targets of internally acting stressors (1,3-β glucan synthase) could help elucidate how TUDCA is rescuing these forms of stress.

The former mechanism we propose here (ER stress priming via CWI activation) is supported by TUDCA's ability to reduce UPR signaling/*HAC1* splicing during Tm treatment (**Figure 4.3**); since both Tm alone and Tm+TUDCA caused a similar amplitude of % spliced *HAC1*, this suggests that Tm entered the cell to a similar extent in both cases. The latter mechanism (in which TUDCA exerts its effects externally only) is supported by TUDCA's ability to decrease cell wall porosity (as measured by fluorescence quenching) (**Figure 4.9**) and by TUDCA's inability to resolve genetic induction of UPR (**Figure 4.4**). This would also potentially explain the concentration-dependent effects of TUDCA's rescue of Tm-induced stress; if the process is based on reducing passive diffusion, a higher concentration gradient could circumvent this. This mechanism may not necessarily support previous literature demonstrating TUDCA's effects in mammalian cells, which lack a cell wall. Yeast CWI components are conserved in mammalian cells as MAPK orthologs, however; Slt2, for example, is an ortholog of the mammalian ERK1/2 (Goshen-Lago et al., 2016). The ERK/MAPK pathway may therefore be involved in TUDCA's mechanism in mammalian systems. Alternatively, TUDCA could exert its effects in mammalian cells by preventing uptake of stressors through the plasma membrane or via transporters. As previously mentioned, another current theory on TUDCA's mechanism of action is that it acts as a chemical chaperone, resolving ER stress by directly assisting in the refolding of misfolded proteins in the ER. Our data, however, contradict this. TUDCA's ability to resolve cell wall-specific stress (as opposed to solely ER stress) and its inability to resolve genetic causes of UPR induction (especially in the case of *scj1*Δ cells, which harbour a deletion in an ER chaperone) do not support the role of TUDCA as a chemical chaperone.

Other bile acids may or may not have similar effects on both ER and cell wall stress; several have been shown to modulate ER stress pathways in widely variable ways. A

non-conjugated precursor to TUDCA, cholic acid, has been shown to decrease ER stress in hepatocytes, adipocytes, and pancreatic β cells (Cummings et al., 2013). On the other hand, treatment of rat hepatocytes with glycochenodeoxycholic acid was shown to induce ER stress and increase apoptosis (Tsuchiya et al., 2006). Lithocholic acid was shown to increase yeast chronological lifespan (Burstein et al., 2012; Goldberg et al., 2010), a process that we have previously demonstrated is heavily dependent on efficient resolution of ER stress through the UPR (Chadwick et al., 2019). This same bile acid was shown to induce ER stress, autophagy, and apoptosis in prostate cancer (Gafar et al., 2016). Much like the existing literature on TUDCA, it appears that other bile acids can regulate ER stress in different ways depending on the cell type and context. Future work in this field could help identify how and why bile acid treatment yields such mixed results.

Overall, this research has made strides in determining TUDCA's mechanism of action and has identified novel interactions with the CWI pathway in our yeast model. Our data also support previous literature which indicated an overlap or compensatory mechanism between stress responses like the CWI pathway and the UPR during multiple kinds of stress exposure. Identifying TUDCA's mechanism of action may also help us better understand the mechanisms of the diseases it appears to treat. This, in turn, could help TUDCA be eventually refined into a more potent and effective therapeutic. This work may also shed light on exactly how and when the numerous stress response signaling pathways in the cell interact, identifying new points of intersection relevant to multiple fields of study.

4.4 Materials and Methods

4.4.1 Strains and cell culture

Strains used in this study are listed in **Table 4.2**; all strains were derived from BY4742 or W303 *Saccharomyces cerevisiae*. For every experiment, cells were thawed from frozen stocks and grown on YPD or selective SC agar plates at 30°C for two days before transferring to liquid culture.

Table 4.2: Strains used in chapter 4

Strain	Description	References
BY4742	*his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mat α*	(Brachmann et al., 1998)
W303	*leu2-3,112 trp1-1 ura3-1 his3-11,15 mat a*	(Pincus et al., 2010)
*ire1*Δ	Same as BY4742 except *ire1*Δ: KAN	Deletion library
*hac1*Δ	Same as BY4742 except *hac1*Δ: KAN	Deletion library
*scj1*Δ	Same as BY4742 except *scj1*Δ: KAN	This study
*spf1*Δ	Same as BY4742 except *spf1*Δ: KAN	This study
*ccw12*Δ	Same as BY4742 except *ccw12*Δ: KAN	This study
*slt2*Δ	Same as BY4742 except *slt2*Δ: KAN	Deletion library
*cnb1*Δ	Same as BY4742 except *cnb1*Δ: KAN	Deletion library
Ire1-D828A	Same as W303 except D828A mutation in *IRE1*	(Chawla et al., 2011)
Bck1-20	Same as W303 except Bck1-20:*trp*	(Lee and Levin, 1992)

4.4.2 Spot assays

Cells were inoculated in 5 mL liquid media in polystyrene snap cap tubes, then grown overnight at 30°C in a rotating drum. OD_{600} of cultured cells was measured using cuvettes and a spectrophotometer. Cells were diluted to a final concentration of OD_{600} 0.2, then 5x serially diluted. Dilutions were spotted onto agar plates and incubated at 30°C for two days before imaging.

4.4.3 Liquid growth assay

Cells were inoculated in 5 mL liquid media in polystyrene snap cap tubes, then grown overnight at 30°C in a rotating drum. OD_{600} of cultured cells was measured using cuvettes and a spectrophotometer. Cells were diluted to a final concentration of OD_{600} 0.15 in a flat-bottom 96-well plate and loaded into a BioTek plate reader in triplicate. Plates were held at 30°C with constant shaking and OD_{600} measurements were taken every 15 minutes.

4.4.4 RNA isolation and northern blot

RNA levels were assessed using northern blot as previously described (Lajoie et al., 2012; Li et al., 2000). Briefly, early-log cells were treated with Tm and TUDCA and cells were pelleted and frozen in dry ice. Total RNA was isolated using hot phenol method (O'Connor and Peebles, 1991). RNA was run on formaldehyde gels and transferred to Nytran Plus membrane. Blots were probed with ^{32}P-labeled oligonucleotide and imaged using 820 Phosphorimager (GE Healthcare, Pittsburgh, PA).

4.4.5 Flow cytometry

Flow cytometry was used to measure fluorescence from fluorescent protein-expressing strains using the BD FACS Celesta and FACS Diva software. Strains were grown in 50 mL flasks to mid-log phase (OD_{600} ~0.5), then treated as indicated (Tm, TUDCA, or both) for indicated time periods before measurement of fluorescence using a PE-TexasRed filter. For time course experiments, samples were loaded in triplicate into 96-well plates and measured using the BD FACS Celesta HTS plate reader system; 20 µL samples were taken at each time point and fluorescence data for a maximum of 10,000 cells were recorded. Between measurements, plates were incubated at 30°C with constant shaking. Median PE-TexasRed-A fluorescence data for each sample were used for analysis. No gates were applied.

4.4.6 Protein isolation and western blot

Protein was isolated using alkaline lysis (Kushnirov, 2000). Mid-log phase cells were treated as indicated (Tm, TUDCA, or both), OD_{600} was measured, and an aliquot of cells

equivalent of OD_{600}=1.0 was taken from each sample. Cells were pelleted then resuspended in 200 µL 0.1 M NaOH and incubated at room temperature for 5 minutes. Cells were pelleted again, then resuspended in 50 µL of sample buffer (4x Laemmeli buffer, water, and 1M DTT) before boiling at 100°C for 3 minutes. Samples were pelleted and the supernatant was transferred to new 1.5 mL tubes.

Proteins were loaded into BioRad TGX Stain-Free gels and separated by gel electrophoresis. Stain-free technology was used as a loading control by activating the gel with UV light for 5 minutes before transfer. Proteins were transferred to nitrocellulose membranes using a BioRad Turbo Blot system and imaged with UV light to obtain final stain-free images. Membranes were incubated at 4°C overnight with anti-PDI1 antibodies (Santa Cruz Biologicals, 1/5000 in 5% skim milk) then probed with secondary antibodies (Licor goat anti-mouse) for 1 hour at room temperature before imaging. Densitometry analysis was conducted using Image Lab software.

4.4.7 Deletion library screens

Using the Singer ROTOR robotic system, frozen stock yeast deletion libraries were thawed and spotted from liquid cultures onto agar plates using 96- or 384-pin pads. Plates were incubated at 30°C for 1-2 days, then re-pinned onto agar plates containing indicated treatments and incubated at 30°C once again. Plates were imaged using a Nikon camera dock. Colony size was quantified using ImageJ, and ratios of growth rescue were calculated by dividing relative colony size on Tm + TUDCA plates by colony size on plates containing TUDCA alone.

4.4.8 Fluorescence quenching assay

Cell wall integrity was assessed using fluorescence quenching assays (Liu et al., 2019). Cells were grown to mid-log phase then treated as indicated (SDS, TUDCA) for 2 hours before staining. Cells and reagents were kept on ice at all times. Cells were washed with cold PBS then resuspended in 200 µL FM4-64 at a final concentration of 50 µM in DMSO, incubated for 3 minutes, and washed with PBS again. Stained cells were imaged immediately using LABTEK (Nunc Inc.) imaging chambers. Bright field and fluorescent images were acquired using a Zeiss Axiovert A1 wide-field fluorescent microscope

equipped with a 560 nm excitation/630 emission (TexasRed) filter, a 63× 1.4 numerical aperture oil objective lens, and a Zeiss AxioCam ICm1 R1 CCD camera. Once initial images were taken, fluorescence was quenched by adding trypan blue to a final concentration of 10 μM and cells were imaged immediately once again. Three fields of view were taken for each sample, before and after quenching.

4.4.9 Cell viability staining

Cells were stained with propidium iodide to detect viability as previously described (Chadwick et al., 2016). A total of 200 μL of each cell culture were added to 1.5 mL tubes, plus two extra samples to serve as positive and negative controls. The positive control was boiled for 10 min at 100°C. All samples were pelleted for 2.5 min. All samples except the negative control were resuspended in 200 μL of phosphate-buffered saline (PBS) containing a 1 in 200 dilution of a PI stock solution (1 mg/mL in H_2O). The negative control was resuspended in 200 μL of PBS alone. The samples were incubated at room temperature for 10 min. Imaging was then performed using the Gel Doc system (BioRad) using the red fluorescent filter. The optical density (OD_{600}) of each sample was determined using a plate reader. Survival rates and integrals were calculated using the ANALYSR program.

4.5 Supplemental Figures

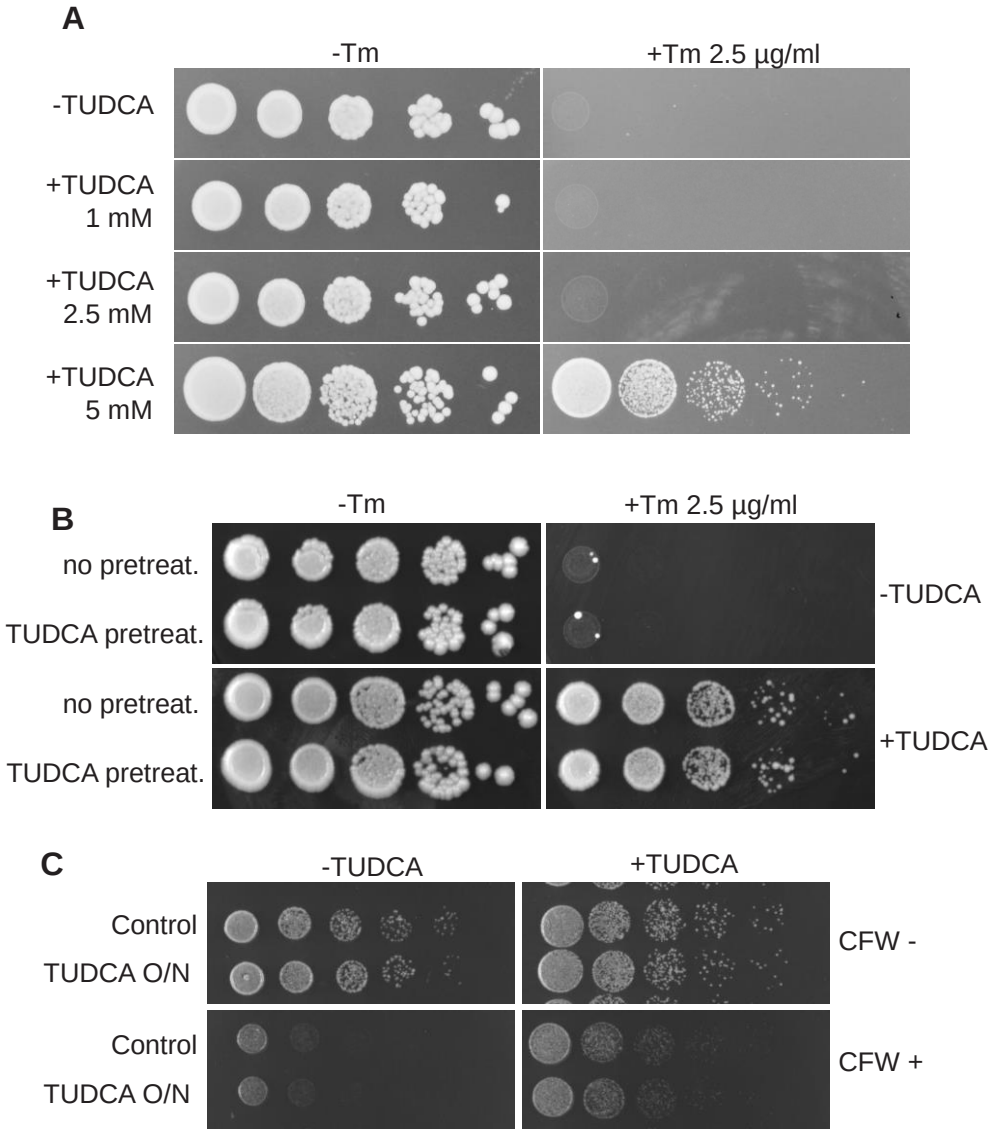

Figure S4.1: Identification of TUDCA concentration's effect on ER stress, and effect of TUDCA pretreatment. A) Wild-type cells were spotted on YPD plates containing 2.5 µg/mL Tm, with a range of concentrations of TUDCA. **B)** Wild-type cells and those treated overnight with 5 mM TUDCA were spotted on YPD plates containing 2.5 µg/mL Tm, with or without TUDCA. **C)** Wild-type cells and those treated overnight with 5 mM TUDCA were spotted on YPD plates with or without the cell wall stressor calcofluor white (CFW), with or without TUDCA. All plates were incubated at 30°C for two days before imaging.

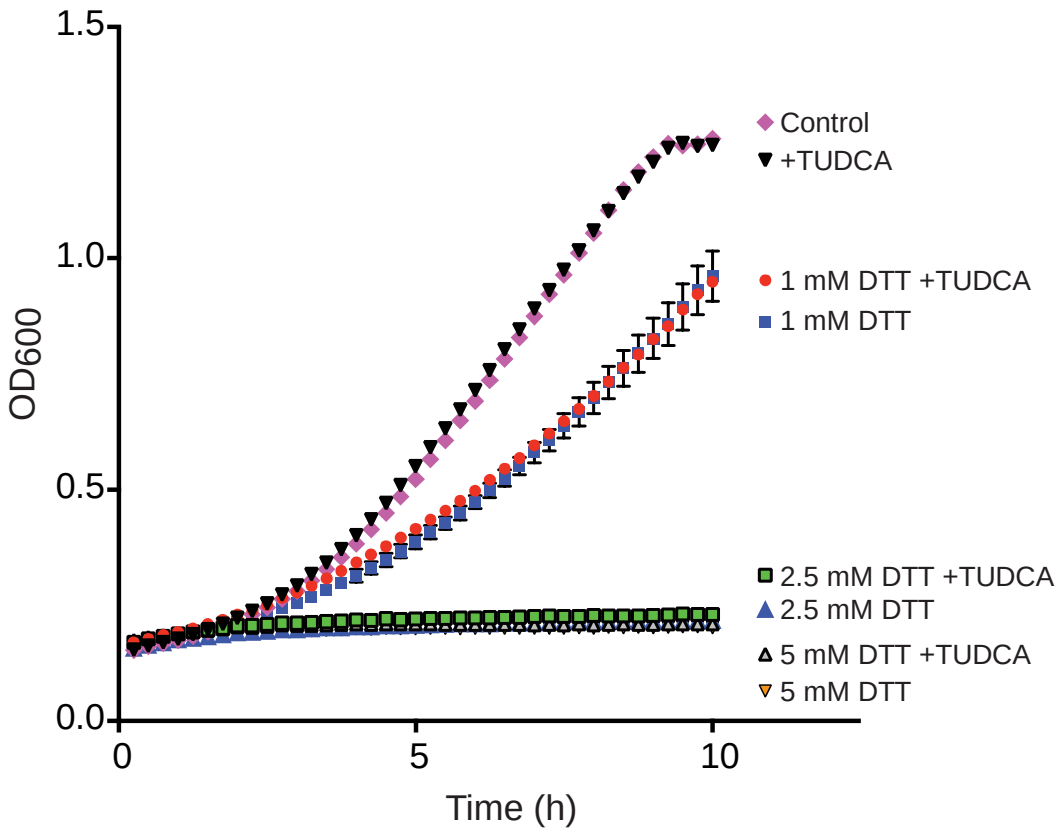

Figure S4.2: TUDCA does not rescue DTT-induced growth defect. Wild-type cells were grown to saturation then diluted to OD_{600} 0.15 in media containing various concentrations of DTT, with or without TUDCA. Cells were loaded into a 96-well plate at 30°C with constant shaking and OD_{600} measurements were taken every 15 minutes for 10 hours (n=3).

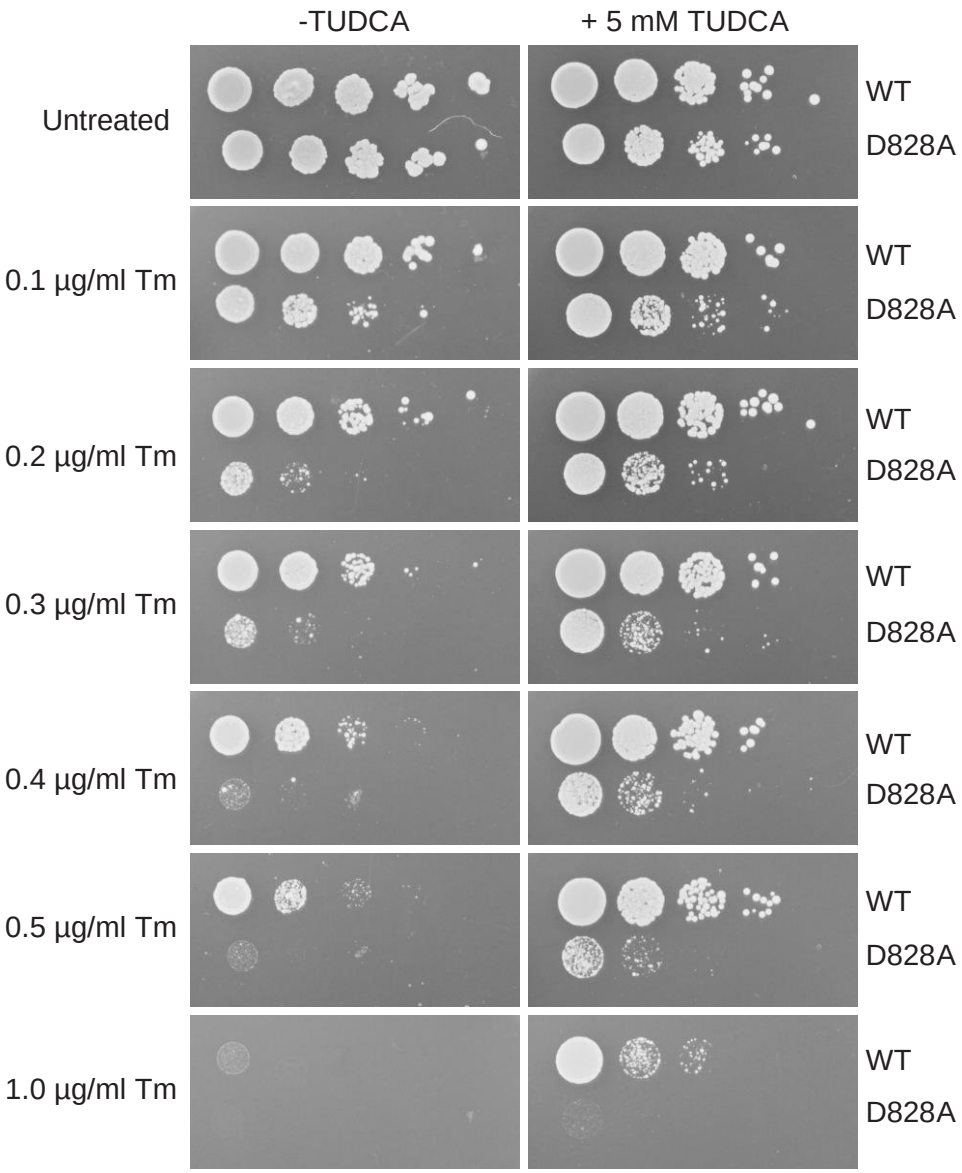

Figure S4.3: TUDCA rescues Tm-induced growth defect of a non-attenuating *IRE1* mutant in a concentration-dependent manner. Wild-type cells and those expressing the mutant Ire1-D828A were diluted to OD 0.2 and spotted in serial fivefold dilutions on plates containing a range of concentrations of Tm, with or without TUDCA. Plates were incubated at 30°C for two days before imaging.

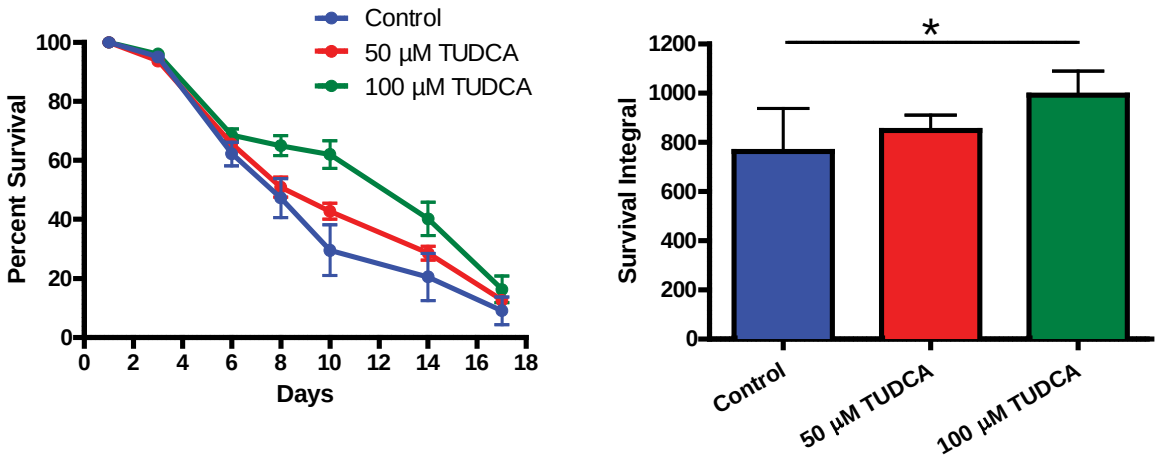

Figure S4.4: TUDCA slightly increases chronological lifespan. Wild-type cells were aged in SC media containing the indicated concentrations of TUDCA. At each time point, cells were stained with propidium iodide to assess viability. Fluorescence data was converted to survival curves and integrals using ANALYSR software (n=5).

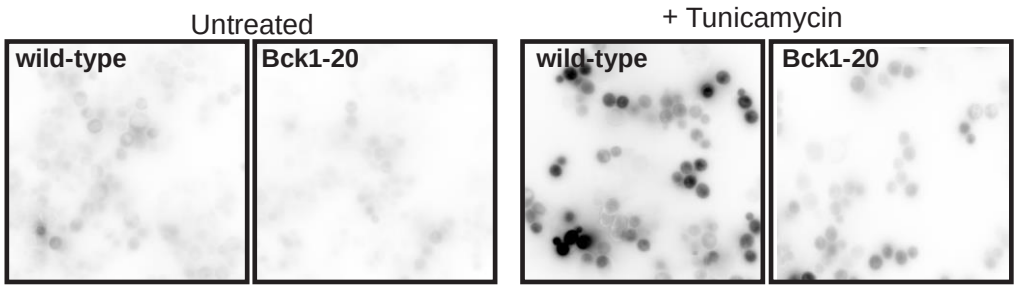

Figure S4.5: Hyperactive cell wall mutant is less sensitive to ER stress. Wild-type and Bck1-20 W303 cells expressing the fluorescent reporter UPR-mCherry were treated with 2.5 µg/ml Tm and assessed by fluorescent microscopy.

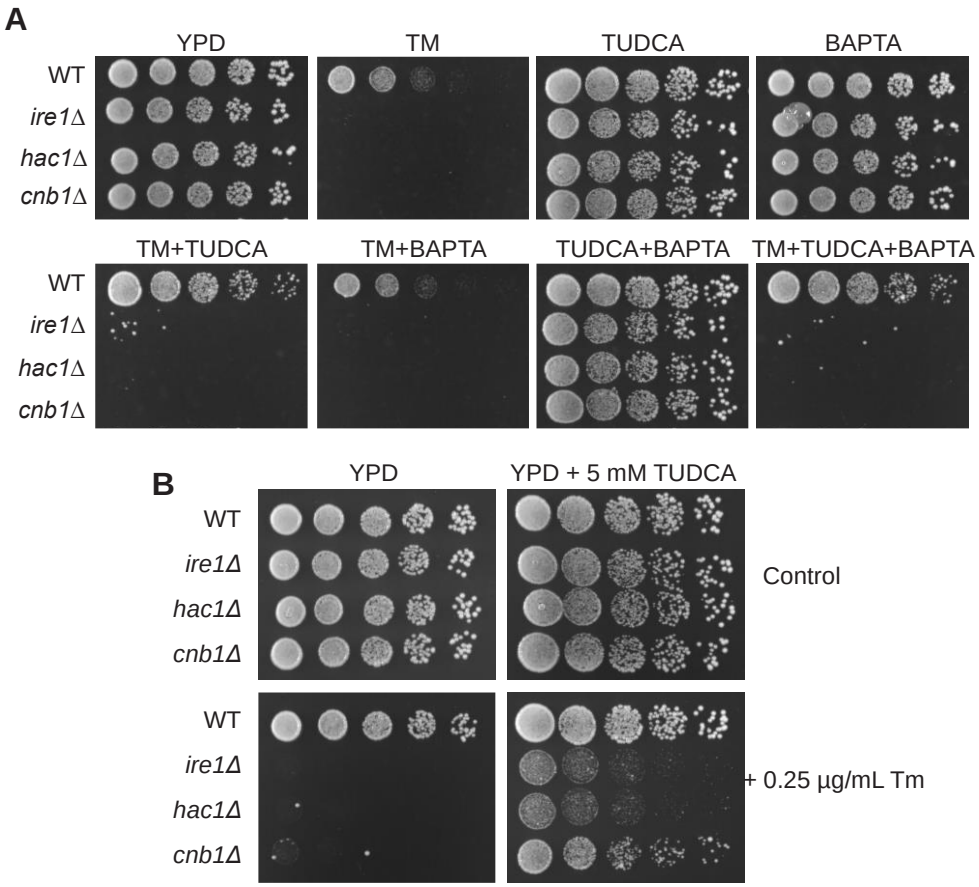

Figure S4.6: TUDCA's rescue of ER stress does not require calcium or calcineurin signaling. A) Wild-type BY4742 cells those with deletions in *IRE1*, *HAC1*, or *CNB1* were grown overnight in SC media, diluted to OD 0.2 and spotted on YPD/SC plates with a combination of Tunicamycin (1 µg/mL), TUDCA (5 mM), and the calcium chelator, BAPTA (25 µM). **B)** The same cells were spotted on plates containing 0.25 µg/mL +/- TUDCA. Plates were incubated at 30°C for two days before imaging.

4.6 Acknowledgements

This study is supported by a project grant from the Canadian Institute for Health Research (CIHR) to PL. SRC holds an Alexander Graham Bell Canada Graduate Scholarship from the Natural Sciences and Engineering Research Council of Canada (NSERC).

4.7 References

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Chapter 5

5 Discussion

5.1 Summary of Major Results

This thesis was designed to address three major research goals: first, we aimed to develop a new software-assisted experimental workflow to quantitatively assess yeast chronological lifespan. Using these and other methods, we next aimed to define how ER stress and the UPR modulate chronological lifespan in a yeast model of aging, and to understand how the unfolded protein response and ER-associated degradation impact ER homeostasis. Finally, using our knowledge of the role of the UPR, ERAD, and ER stress gleaned from this previous work, we aimed to apply these concepts to a clinically relevant problem: modulating ER stress toxicity pharmacologically. Specifically, we wanted to decipher the mechanisms underlying the ability of the bile acid TUDCA to alleviate ER stress.

5.1.1 Chapter 2: A toolbox for rapid quantitative assessment of chronological lifespan and survival in *Saccharomyces cerevisiae*

The research presented in Chapter 2 aimed to both improve the methods to study the yeast aging process, and also contribute to the existing research in the field. It was demonstrated that fluorescent viability dyes (such as propidium iodide or SYTOX green) can be used to assess CLS and reliably recapitulate aging data obtained using more traditional methods, such as spot assays. These viability dyes can then be assessed using multiple means depending on the equipment available: a UV transilluminator (Gel Doc), an automated cell counter, or a standard 96-well plate reader. Importantly, these methods allowed for rapid, reliable quantitative measurement of yeast CLS, much like the data obtained from colony forming unit (CFU) assays or flow cytometry, while being higher throughput and faster (in the case of CFU assays) and remaining accessible to laboratories with limited funding (in the case of flow cytometry). In addition, we also developed an accompanying software program to assist in data analysis. The Python-based ANALYSR program allows users to quickly adjust raw fluorescence data to cell

density, apply positive and negative controls, and normalize data to generate survival curves with day 1 of aging set to 100% survival. Using a well-defined model of aging and lifespan extension, caloric restriction, we were able to show that these methods could complement or replace existing measures of CLS. Finally, as an example of the applications of these methods and software outside the realm of CLS, we demonstrated that the concurrent use of both traditional and new methods could allow researchers to add nuance to their findings. We found that differences in cell survival (as measured directly through viability staining) and cell division (as measured through regrowth assays, such as spot assays) could be used to identify mechanisms of cell death; cells with deletions in three major heat shock proteins showed a significant growth defect after heat stress, but showed little to no change in PI staining. This indicates that the cells may have stopped dividing, but were not necessarily dead, or that the mechanism of cell death after heat stress was one that did not compromise the plasma membrane (e.g. more similar to apoptosis than necrosis). Through the development of new techniques to assess chronological lifespan in yeast, high throughput and quantitative data on survival rates has been made more accessible. This, in turn, allows the expansion of aging research into more and more variables, which can be easily assessed and compared using these methods and accompanying software. Combined with the existing, traditional methods of measuring yeast lifespan, researchers are able to further validate their research and add power to the results they obtain (Chadwick et al., 2016).

5.1.2 Chapter 3: A functional unfolded protein response is required for chronological aging in *Saccharomyces cerevisiae*

The development of the methods outlined in Chapter 2 allowed a more thorough investigation into the impact of aging and endoplasmic reticulum stress response pathways upon yeast chronological aging, which was explored in Chapter 3. This research presents evidence that lipids, aging, and other ER stressors play a role in modulating the cellular response to stress, both through ER-associated degradation and the Unfolded Protein Response. Defects of ERAD and the UPR, in turn, lead to defects in yeast chronological aging, as the cells are less able to adapt to new forms of stress. One aspect of this research demonstrated the powerful role for lipid content and concentration

in the growth media (specifically inositol) in aging, in wild-type cells as well as those with compromised UPR. In particular, low inositol media was shown to induce the UPR even in wild-type cells, which could have important implications for how yeast aging research is currently conducted. The use of low inositol media is a standard practice, and this media has been used to conduct many high throughput screens and other experiments. Because low inositol itself appears to be a stress source for yeast cells, it may make the assessment of other stressors (aging, DTT, etc.) more difficult by confounding the results. Two other potential environmental modifiers of CLS, including pH and caloric restriction, were shown to alter CLS (as previously described), but did so independently of the UPR. In addition, we showed that the specific link between the chaperone protein Kar2 and the UPR (through the UPR response element in its promoter) is essential for adaption to, and survival during, ER stress. The mere presence of Kar2 was not sufficient to respond to ER stress, but its decreased abundance (through use of the DAmP allele) also impaired chronological aging. This could indicate that both the continued presence and UPR-related increased in Kar2 abundance are essential in the UPR's ability to resolve stress, both in response to aging and the presence of small molecule stressors (such as Tm). Importantly, this research also suggests a role for ERAD in aging and offers new insights into ERAD itself. Deletion of either of the E3 ubiquitin ligases involved in ERAD's different branches (based on the cellular location of the degron tag) did not affect yeast CLS, but double deletion of both ligases shortened CLS significantly. Of the two E3 ubiquitin ligase complexes, the Hrd1 complex has been shown to recognize proteins with luminal or membrane-oriented degrons (ERAD-L or M), whereas the Doa10 complex recognizes proteins with cytoplasmic degrons (ERAD-C) (Carvalho et al., 2006; Crowder et al., 2015; Rubenstein et al., 2012). However, our data indicate redundancy between these pathways; when one is non-functional, the other appears to compensate. When both are non-functional, ERAD becomes impaired and leads to shortened CLS. This redundancy has been suggested before (Rubenstein et al., 2012), and we were able to directly demonstrate that functional ERAD (with either Hrd1 *or* Doa10 present) is sufficient to prevent the short-lived phenotype caused by misfolded protein accumulation in cells lacking both ERAD branches. The results presented in this chapter can be used to study topics or diseases at the intersection of aging and misfolded

proteins/ER stress, including the age-related neurodegeneration seen in Alzheimer's and Huntington's disease. These data also offer insights into modulating ER stress in general; by understanding the genes and pathways important for the tolerance of chronological aging, an ER stressor, these genes and pathways could be used as targets to alter tolerance for other forms of stress (Chadwick et al., 2019).

5.1.3 Chapter 4: Regulation of ER stress resistance by tauroursodeoxycholic acid in yeast

The research presented in Chapter 4 builds upon that of previous chapters; using our understanding of the importance of the UPR and related protein quality control pathways in ER stress resistance, we aimed to identify the mechanism of a compound posited to modulate these: TUDCA. TUDCA has been repeatedly linked to changes in UPR signaling, and we therefore hypothesized that it may alter stress tolerance by altering the targets or induction level of the UPR (Berger and Haller, 2011; Drack et al., 2012; Engin et al., 2013; Rivard et al., 2007; Seyhun et al., 2011; Uppala et al., 2017). We also aimed to explore the possibility that it acted as a chemical chaperone, directly improving protein folding, degradation, or trafficking (de Almeida et al., 2007; Loo and Clarke, 2007; Upagupta et al., 2017). We found that TUDCA was indeed able to rescue multiple forms of stress (including ER stress), but unexpectedly, it did so independently of the UPR. TUDCA reduced the effect of Tm on N-linked glycosylation and reduced UPR signaling upon Tm exposure, but was still able to do so in the absence of *IRE1*, the major stress sensor and UPR effector in yeast. It was not able to rescue genetic causes of protein misfolding and ER stress, as demonstrated by deletions in *SPF1* and *SCJ1*, two ER-resident proteins which generate high levels of ER stress when deleted. Together, these data indicated a role for TUDCA in negating the effects of Tm on the UPR, not directly affecting the UPR or refolding misfolded proteins as a chemical chaperone would, contradicting existing literature and rejecting our own hypotheses. Instead, we demonstrated a surprising role for TUDCA in modulating the Cell Wall Integrity pathway; TUDCA was able to alleviate cell wall stress and appeared to physically alter cell wall morphology/porosity. We offer two potential mechanisms to explain these data: First, activation of CWI signaling may prime or pre-condition cells, increasing their

tolerance for other stressors (such as Tm) while also leading to changes in the cell wall. Alternatively, changes in cell wall composition or porosity could physically reduce Tm's entry into the cell. This research has important implications for research on chemical chaperones and protein folding: our data indicate that TUDCA is not, in fact, a chemical chaperone, but is still able to modify ER stress (and potentially the ER protein folding environment) in a previously undetected way. These data offer new information about TUDCA's mechanism for alleviating ER stress and also identify new potential methods for indirectly targeting ER stress for therapeutic purposes.

5.2 Modelling and Modulating the UPR

While *S. cerevisiae* is an effective tool for studying and modelling the UPR, numerous other model organisms have been used for similar purposes. For example, the fission yeast *Schizosaccharomyces pombe* can be used to study the RIDD branch of Ire1's signaling pathway in isolation; *S. cerevisiae* lack this function. *S. pombe*, however, lack *HAC1* splicing activity, allowing the separate study of different aspects of the mammalian IRE1's multiple functions (Kimmig et al., 2012; Li et al., 2018). *S. pombe* have been used for aging studies as well; regulation of chronological lifespan in fission yeast has been thoroughly studied (Roux et al., 2006; Zuin et al., 2008). Replicative lifespan has not been covered as extensively in *S. pombe*, primarily due to the fact that it is not as simple to measure as it is in *S. cerevisiae*. While budding yeast divide asymmetrically with obvious morphological differences between the mother and daughter cells, fission yeast divide equally and with fewer differences (Barker and Walmsley, 1999; Lin and Austriaco, 2014; Steffen et al., 2009). Studying this form of cell division can be useful, however, since it is more similar to that of mammalian cells in comparison to budding, and involves conserved cell division mechanisms such as mitochondrial inheritance (Chiron et al., 2007; Roux et al., 2010). UPR models exist in higher organisms as well. In *Drosophila melanogaster*, both the IRE1/XBP1 and PERK/ATF4 pathways of the mammalian UPR are conserved; *Drosophila* also express a form of ATF6 which shares sequence homology with the mammalian form, but evidence that it acts as a functional homologue is limited (Lindström et al., 2016). *Drosophila* models have been used to study aging, HSR, and UPR in disease models, such as Alzheimer's

and retinal degeneration (Bai et al., 2013; Marcora et al., 2017; Morrow et al., 2004a, 2004b; Ryoo et al., 2007). Another invertebrate model option is *Caenorhabditis elegans*, which can be particularly powerful since all three mammalian UPR pathways are conserved, allowing for simpler study of the UPR as a whole (Calfon et al., 2002; Shen et al., 2001). As a result, *C. elegans* has been used to model many UPR- and misfolded protein-related diseases, especially neurodegenerative diseases like Huntington's and Parkinson's (Brignull et al., 2006; Parker et al., 2004; Scheper and Hoozemans, 2015; Ved et al., 2005). Mammalian models are, of course, also common; a wide variety of knockout and conditional knockout mice have been generated, each lacking a different component of the UPR signaling pathway (Bommiasamy and Popko, 2011). Mice can also be engineered to express or overexpress disease-causing misfolded proteins (such as amyloid precursor protein, causing Alzheimer's disease symptoms, or expanded Htt protein to model Huntington's disease), allowing the study of the UPR in more directly applicable disease models (Bradford et al., 2010; Hashimoto et al., 2018; Lee et al., 2010). Multicellular organisms (including mammals and invertebrates) can also be particularly useful for studying the concept of cell nonautonomous UPR induction, wherein stress in one tissue or subset of cells can lead to systemic, whole-organismal activation of stress responses (Frakes et al., 2020; Mahadevan et al., 2011; Taylor et al., 2014). In *C. elegans*, four distinct glial cells were found to control UPR induction (and, by extension, lifespan) in distal cells; when spliced *XBP1* mRNA was overexpressed in these cells, it led to increased tolerance to ER stress and protein aggregation throughout the whole organism, mediated by neuropeptides packaged in vesicles (Frakes et al., 2020). Similarly, a study in mice showed that ER stress can be "transmissible"; macrophages grown in conditioned media from ER-stressed tumour cells activated ER stress-induced pathways of their own, upregulating genes such as *XBP1* and *GRP78* (or BiP, the mammalian homologue for Kar2) (Mahadevan et al., 2011). Another mouse study showed that ER stress was transmissible between cells of the central nervous system (Sprenkle et al., 2019), a concept which has been proposed to explain the multicellular dysfunction seen in some neurodegenerative diseases, such as ALS (Ilieva et al., 2009). The influences of systemic ER stress and UPR activation can therefore be

very powerful, and while this field is still developing, understanding cell nonautonomous UPR will likely be important for targeting the UPR in disease.

As discussed in Chapter 1 and 4, previous studies have attempted to find ways to modulate the UPR and ER stress for therapeutic purposes. There are two primary ways to achieve this: first, one can target misfolded proteins themselves, decreasing the misfolded protein burden in the ER lumen by improving protein refolding or degradation. Lessened misfolded protein burden thereby leads to lessened UPR activation. Secondly, one could target the UPR signaling pathway itself, reducing UPR activation (or altering UPR targets) independent of misfolded protein burden. Chemical chaperones such as 4-PBA or glycerol can be used for the former, and UPR effector protein inhibitors, such as those targeted against IRE1, PERK, or ATF6, can be used to achieve the latter. These methods have been used for several purposes (including attempts to both increase and decrease ER stress-mediated cell death) and have had mixed efficacy and highly variable results in the literature. This is not necessarily surprising, given the UPR's ability to lead to either adaptive or maladaptive responses; for example, cancer cells may increase their survival or chemoresistance through increased UPR signaling (Rouschop et al., 2013; Tay et al., 2014), while UPR overinduction leads to ER stress-induced apoptosis in models of retinal degeneration (Ghosh et al., 2014). These two systems present with similar etiology, but the goal of UPR modulation in treating them is quite different (e.g. reducing UPR to cause cell death in the case of cancer, vs. reducing UPR to prevent cell death in retinal degeneration). It is therefore highly important to understand how the UPR functions in different circumstances or disease models when attempting to modulate it. The research presented in Chapter 3 aimed to do this: these data offer new information on the aging cell and the role the UPR plays in this process. We have identified the key players in determining yeast lifespan (namely Ire1, Kar2, and Hrd1/Doa10), and we were able to separate the UPR from other mechanisms of lifespan extension. Previous research had shown that yeast's other form of lifespan, replicative lifespan, was different in this regard. Defective UPR or prolonged ER stress were not shown to reduce RLS; in fact, deletions of downstream UPR targets were shown to slightly extend RLS, and deletion of the major UPR effectors *IRE1* and *HAC1* had no effect (Labunskyy et al., 2014). Our data show that Ire1 and Hac1 were absolutely required for normal chronological aging, but

that deletions in these did not affect lifespan extension by caloric restriction or pH buffering. Caloric restriction has been proposed to extend yeast RLS (and the lifespan of numerous other organisms, such as *C. elegans*) through sirtuins (such as Sir2) which regulate a wide variety of cellular processes (Lin et al., 2000; Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). In contrast, the role of sirtuins is not clear in CLS extension; some studies have demonstrated that deletion of *SIR2* did not affect CLS extension by caloric restriction (Fabrizio et al., 2005; Smith et al., 2007). On the other hand, pH buffering of growth media has been shown to significantly extend yeast CLS (Burtner et al., 2009), but has little to no effect on RLS (Wasko et al., 2013). This has been suggested to be related to the caloric restriction mechanism of lifespan extension, wherein decreased glucose metabolism results in lessened media acidification during aging, but this would not explain why caloric restriction still extends RLS yet pH buffering does not (Wasko et al., 2013). Different sets of genes may therefore be involved in the metabolic demands of yeast growth and division (RLS) vs. long-term survival in a non-dividing state (CLS), offering new insights into the role of sirtuins, metabolism, and aging overall. Given that RLS is often used as a model for aging in dividing cells and CLS generally models aging of post-mitotic cells, these findings may also apply to mammalian cells with different metabolic demands and activities.

The fact that *ire1*Δ cells had severe aging and growth defects under normal growth conditions but were indistinguishable from their wild-type counterparts under pH buffered/calorie restricted conditions also makes it clear that ineffective UPR can be circumvented. This is supported by our data in Chapter 4, wherein we show that TUDCA was able to rescue ER stress even in *ire1*Δ cells, which are already hypersensitive to stress. This likely occurs through the activation of other stress response pathways, such as CWI, which compensates for the loss of UPR. This represents a new potential method for future therapies; when neither the misfolded protein burden nor the UPR itself can be modulated, targeting other stress response pathways may be an option. This could be important since the UPR plays a wide variety of roles in both health and disease, and directly inhibiting or increasing it could have numerous off-target effects.

It would also be valuable to further study the wide variety of mechanisms of UPR activation that exist when attempting to modulate it. As previously mentioned, it has been shown that different forms of UPR activation can lead to different transcriptional programs. Lipid bilayer stress and inositol depletion have been repeatedly shown to activate the UPR independently of misfolded proteins (Ho et al., 2019; Koh et al., 2018; Promlek et al., 2011). While UPR induced by ER vs. lipid bilayer stress lead to transcription of many of the same genes, the two have been shown to activate ATF6 differently and thereby alter its transcriptional targets, leading to different cellular responses in order to resolve different forms of stress (Fun and Thibault, 2020; Tam et al., 2018). It is therefore possible that the same is true of other stressors that have been previously associated with UPR activation. While many stressors have been assumed to lead to UPR activation by indirectly causing proteotoxic stress (e.g. heat stress causing proteins to unfold) there may actually be many other distinct transcriptional programs initiated by the UPR in different circumstances. Identifying differences in the genes involved in responding to various stressors (via the UPR or otherwise) will be essential in understanding the UPR as a whole, as will identifying the other cellular pathways with which it converges.

5.3 Multiple Pathways Intersect to Regulate ER Stress Sensitivity

One major theme of the research conducted in this thesis is the intersection between ER stress, the UPR, and other stress response and cellular quality control pathways. While ER stress triggers UPR activation, it also can lead to the activation of other pathways, such as the Heat Shock Response (Liu and Chang, 2008), and can itself be induced downstream of other stressors, such as oxidative stress (Guo et al., 2009). ER stress can be resolved via the downstream effects of the UPR (e.g. expanded ER lumen, increased chaperone proteins, decreased protein translation, protein degradation), but can also be resolved by other quality control processes. These processes include ERAD, which selectively degrades misfolded or unfolded proteins in the ER, as well as autophagy, which can generally or selectively degrade organelles and other cellular contents upon ER stress induction (Duennwald and Lindquist, 2008; Friedlander et al., 2000; Yorimitsu

et al., 2006). A specific branch of autophagy, dubbed “ER-phagy”, has been shown to become activated by ER stress both through UPR signaling and independently of the UPR; it can degrade excessive ER membrane caused by UPR-triggered ER expansion, and it may also play a role in sequestering and degrading toxic misfolded proteins from the ER (Bernales et al., 2006; Ogata et al., 2006; Schuck et al., 2014; Yorimitsu et al., 2006). Autophagy may also play a role in ER stress-induced apoptosis; it has been hypothesized to counterbalance apoptotic signals, delaying or preventing apoptosis in favour of cell survival (Ogata et al., 2006; Ravikumar et al., 2006). There is also evidence that apoptotic proteins, such as Bcl-2 family proteins, and autophagic proteins, including Beclin-1 (ATG6 in yeast) may interact in different ways depending on the cellular circumstance, either leading to autophagy and cell survival or apoptosis (Bassik et al., 2004; Rubinstein and Kimchi, 2012; Wei et al., 2008; Wirawan et al., 2010).

Another major modulator of ER stress and ER stress-induced apoptosis is calcium flux. When ER stress is prolonged or unresolved, calcium released from the ER into the cytosol during stress can be taken up by the mitochondria, activating intrinsic apoptotic pathways (Hetz, 2012; Lam et al., 1994). As mentioned in Chapter 1, the ER stress inducer thapsigargin works by depleting ER calcium levels, leading to UPR induction and eventually cell death (Földi et al., 2013; Lytton et al., 1991). ER Ca^{2+} plays several important roles in normal proteostasis as well as cell death; the ER resident chaperone BiP (Kar2 in yeast), for example, requires high Ca^{2+} levels for its ATPase activity, and has also been shown to regulate ER Ca^{2+} itself (Lièvremont et al., 1997; Ma and Hendershot, 2004). In fact, disruption of ER Ca^{2+} can lead to aberrant chaperone protein redistribution or secretion, reducing the ER’s capacity to resolve unfolded proteins (Booth and Koch, 1989; Suzuki et al., 1991). Calreticulin and calnexin are ER-resident chaperones that both assist with the proper folding of newly synthesized proteins and glycoproteins, and also buffer ER Ca^{2+} stores (Michalak et al., 2009; Tatu and Helenius, 1997). For these reasons, calcium flux is an important factor in protein folding and ER stress, as well as cell fate. In yeast, calcium released into the cytosol during ER stress can activate multiple calcium-dependent signaling pathways, which restore calcium homeostasis and avoid cell death, known as the Calcium Cell Survival (CCS) signaling pathways (Bonilla and Cunningham, 2003; Bonilla et al., 2002; Locke et al., 2000).

Increased cytosolic calcium leads to activation of calcineurin signaling, a protein phosphatase which dephosphorylates the transcription factor Crz1, which activates genes containing a calcineurin-dependent response element (CDRE) in their promoter (Bonilla and Cunningham, 2003; Bonilla et al., 2002; Yoshimoto et al., 2002). Genome-wide analysis of calcineurin-dependent targets revealed that many of these upregulated genes are involved with small molecule transport and ion homeostasis, as one might expect from a calcium-dependent signaling pathway. However, several are also involved with other processes, such as lipid and sterol metabolism and, interestingly, cell wall synthesis and maintenance via CWI signaling (Yoshimoto et al., 2002).

Calcineurin and calcium-dependent signaling are thoroughly intertwined with CWI pathways. For example, one target of CWI signaling is *GSC2/FKS2*, a subunit of 1,3-β glucan synthase which produces β1,3-linked glucan polymers, a major structural component of the cell wall (Klis et al., 2002; Levin, 2005). *GSC2* is also induced during exposure to high extracellular concentrations of Ca^{2+} through calcineurin signaling (Bonilla and Cunningham, 2003; Garrett-Engele et al., 1995). Calcineurin has also been identified as an essential protein for cellular growth in high osmotic stress environments (Garrett-Engele et al., 1995), further suggesting a role in CWI maintenance. In fact, cells with defective CWI genes (including deletions in *PKC1* or *SLT2/MPK1*) exhibit a more severe growth defect when lacking functional calcineurin, and a less severe growth defect when calcineurin is constitutively active (Garrett-Engele et al., 1995). Despite its connection to CWI, our studies could not identify a role for TUDCA in calcium-related pathways; calcium depletion did not affect TUDCA's ability to rescue ER stress, nor did deletion of the calcineurin subunit *CNB1*. Given the overlap between CWI and calcium, future work on TUDCA's mechanism of action may still reveal an interaction with this pathway, though perhaps in a different model system or via a different transcriptional program.

The numerous connections between ER stress, the UPR, CWI, and other biochemical pathways can confound experimental results but can also offer interesting possibilities for modulating one pathway using another. For example, one group has shown that inhibition of the UPR can be used to prevent the induction of ER stress-mediated autophagy,

thereby preventing autophagy-mediated adaptation and chemoresistance in pancreatic cancer (Thakur et al., 2018). Calcium influx from the plasma membrane and activation of calcineurin have both been shown to occur when misfolded proteins accumulate in the ER, and loss of calcineurin genes causes enhanced sensitivity to Tm (Bonilla et al., 2002). The data presented in Chapter 4 offer the potential of a similar approach: targeting CWI signaling in order to modulate ER stress and the UPR. Understanding these points of contact between the numerous adaptation and stress response pathways will likely be an important factor in understanding their individual roles in normal cell function and in disease.

5.4 Experimental Limitations

It is important to note that, while yeast is a powerful model through which to study aging (and other processes), it is not without its limitations. Yeast certainly does recapitulate many of the aspects seen in mammalian aging, and many of the relevant pathways are conserved. However, yeast are a simpler organism than mammals, and simplification of these pathways may make some yeast research difficult to translate to mammals (Carmona-Gutierrez and Büttner, 2014). In addition, yeast (as a single cell model) cannot fully capture the complexities of the biochemical events occurring in whole organisms, which would be important to understand when attempting to study ER stress or UPR-related disease. Yeast have, however, been the source of many important discoveries that have later been translated to mammalian and animal models, including caloric restriction, (Colman et al., 2009; Jiang et al., 2000), sirtuins (Mostoslavsky et al., 2006; Sinclair and Guarente, 1997), and the TOR pathway (Heitman et al., 1991; Kapahi et al., 2004; Vellai et al., 2003; Wilkinson et al., 2012). Yeast models of protein homeostasis, quality control, and aging have also been thoroughly validated (Denoth Lippuner et al., 2014). While the Ire1 branch of the UPR is highly conserved in yeast, the influence of the ATF6/PERK pathways cannot be studied in this model system. This could have implications for studying the actions of UPR-influencing compounds, including chemical chaperones or TUDCA used in Chapter 4, which may exert effects through these alternate branches of the UPR. Another experimental limitation of Chapters 3 and 4 could be the role of glycerol as a potential chemical chaperone; it was used to assess mitochondrial

respiration in *ire1*Δ cells in Supplemental Figure 3.3. While it is unlikely that glycerol hid the effects of defective respiration in *ire1*Δ cells through to its chemical chaperone activities (since the cells were forced to use it as a carbon source), this could still be a confounding factor and must be acknowledged. In addition to differences in the UPR- and aging-related processes in yeast vs. mammals and other metazoans, yeast also have a cell wall and resultant cell wall integrity pathways that must be accounted for. As previously mentioned, though metazoans do not have cell walls, the MAPK signaling pathway that regulates CWI in yeast is highly conserved; both metazoan and mammalian cells have Slt2 homologs in the form of ERK1/2 (Caffrey et al., 1999; Chen et al., 2005; de Nobel et al., 2000; Truman et al., 2007), and *C. elegans* have osmotic stress-sensing pathways that involve yeast CWI homologs, such as Pkc1 (Wheeler and Thomas, 2006). Findings in cell wall-related pathways in yeast may therefore still have implications for other model systems. In-depth analysis of changes in gene expression (such as through RNA sequencing) upon treatment with TUDCA would need to be conducted on both yeast and metazoan models in order to confirm that our findings translate across model systems. It is important, then, to specify that the research conducted here in a yeast model may provide useful information on the basic mechanistic framework of the pathways being studied, but may not translate directly to a disease model without adaptation.

5.5 Future Directions

In terms of long-term future directions, the research detailed in Chapters 2-3 could likely be adapted to other models of ER stress, protein misfolding, and aging. This could include expansion into disease models, such as Huntington's disease (HD). In mouse models, for example, the HD phenotype has been shown to be modulated by lipids (Valenza et al., 2005). Expansion of this research into *C. elegans* would also be feasible for both studying aging itself as well as neurodegenerative disease pathogenesis, in which the process has been well-characterized and can be easily studied (Li and Le, 2013). Future research could also include examination of mammalian cells to assess similarities and differences in the results presented here, or patient samples, which could be used to analyze the biochemical changes that occur during aging. Mass spectrometry, for example, could be used to analyze lipid composition (among other factors) to identify

alterations associated with impaired aging. Additionally, this research could be expanded into other aspects of cellular stress responses, such as the heat shock response (HSR), as it has been shown that the addition of HSR proteins (such as HSF1) to cells with deficient UPR (such as *ire1*Δ) can rescue their growth defect (Liu and Chang, 2008). As certain domains of Ire1 have been shown to be important for responding to proteotoxic stress vs. lipid stress (Ho et al., 2019; Tam et al., 2018), it would also be interesting to determine whether specific domains of Ire1 are required for chronological aging.

In Chapter 4, we identified new mechanistic insights into TUDCA's modulation of ER stress. Future work in this area could include validation of TUDCA's effect on CWI homologues/orthologues in mammalian cells, such as examining activation of the ERK1/2 pathway (Goshen-Lago et al., 2016). Deeper examination of changes to cell wall morphology upon TUDCA treatment may also be valuable; electron microscopy of mammalian cells has shown no significant morphological changes when treated with TUDCA, but yeast cell wall structure has yet to be examined (Xie et al., 2002). The findings in Chapters 3 and 4 could be combined to study TUDCA's ability to ameliorate ER stress in disease models; our group currently works with a yeast model of HD, which TUDCA may be able to rescue (Jiang et al., 2019, 2017). Other yeast models of disease could be used or developed for this purpose as well. In addition, studies to determine intracellular or intraorganelle concentrations of Tm and TUDCA could be used to validate our hypothesis that TUDCA may not enter the cell to exert its effects. Finally, future work could address whether other bile acids (or compounds that are chemically similar to TUDCA) have similar effects on CWI and UPR signaling. Identification of the chemical properties important for TUDCA's mechanism will be essential in optimizing it as a drug or therapeutic and may also offer more information on the targets with which it can bind or interact, which in turn could offer new possibilities for its use in other diseases (potentially outside the realm of ER stress).

5.6 Significance and Overall Conclusions

Overall, this research offers novel information on, and the opportunity to explore, many different facets of the complex relationship between proteostasis, ER stress, and aging. We have uncovered new differences in replicative vs. chronological aging, which will be

significant in understanding the UPR's role in different cellular states; understanding the "type" of UPR involved in a given system or disease etiology will be essential in attempting to target it for therapeutic purposes. Here, we have successfully identified the key effectors of the UPR's response to aging in non-dividing cells (undergoing chronological aging), which could have broad-reaching implications for aging-related neurodegenerative diseases. For example, attempting to target the systems involved in caloric restriction-mediated lifespan extension may have little to no effect on diseases with shortened lifespan and aberrant UPR in non-dividing cells, since we have discovered a separation of these pathways. These findings also contribute to our basic science knowledge of the aging process itself and how it can change cellular functions. Deepening our understanding of the signaling pathway components most important for tolerance of chronological aging will give us insights into the fundamental aspects of cellular response to stress. These findings could then be tested with other systems and other stressors to identify similarities and differences; for example, do these findings translate to higher organisms? Or other forms of stress, such as oxidative stress or heat/cold shock? If so, or if not, why?

We have also identified new evidence that one can indirectly target the UPR. We have shown that TUDCA can reduce the effects of stressors on misfolded proteins in the ER, potentially without ever directly contacting the organelle. The connection we have drawn between the UPR and CWI is a relatively novel one and could have important implications for how the UPR and other seemingly unrelated pathways interact, furthering multiple fields of study. This information could also be used to develop new treatments for UPR-related diseases, or offer the chance to apply existing CWI-related therapies to these diseases. For example, as previously mentioned, the mammalian homologue for the yeast CWI gene *SLT2* is *ERK1/2*. Inhibitors of ERK1/2 for other purposes, such as cancer treatment, have already been developed (Yang et al., 2019). These drugs could have potential in sensitizing cancer cells to ER stress by inhibiting this MAPK pathway. ERK1/2 agonists have also been developed (Wang et al., 2019). These therapeutics could have applications in diseases with increased ER stress sensitivity, such as neurodegenerative diseases; increasing signaling through this pathway could protect against misfolded protein stress. In addition, the data presented here could offer new

information on many of these drugs' mechanisms of action, offering a new avenue for further development and new markers to assess their efficacy (e.g. UPR-related genes and proteins, such as *XBP1* or BiP expression). We could also draw conclusions about the chemical properties that allow TUDCA to alter ER stress sensitivity. Studies have been conducted on the chemical and biochemical aspects of chemical chaperones (Brown et al., 1996; Upagupta et al., 2017), but as we have demonstrated, TUDCA is unlikely to fall in this category. However, TUDCA's steroid-like structure may contribute to its ability to insert into the cell wall or plasma membrane, as it has been shown to increase membrane fluidity in chondrocytes (Arai et al., 2019). Future structural studies on TUDCA and other bile acids may shed further light on the results described in Chapter 4.

The studies presented here represent new advances in our ability to study cell viability over the course of aging in a high throughput manner (Chapter 2), our understanding of the role of UPR signaling pathway components in aging (Chapter 3), and the potential for modulating the UPR and circumventing defective UPR for therapeutic purposes (Chapter 4). The field of proteostasis and cellular stress responses is growing, both in terms of fully characterizing the signaling pathways involved and creating drugs to target these pathways. These data (and this thesis as a whole) will hopefully advance the field and offer new directions for future growth.

5.7 References

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Appendices

Appendix A: Usage of ANALYSR (Analytical Algorithm for Yeast Survival Rates)

Two Python scripts have been developed to aid in data analysis for the Lajoie Lab. This document will discuss the implementation of the scripts, the algorithms they employ, and how data is to be structured so as to be used be properly used by the scripts. This protocol was published in Traffic as a supplemental file:

Chadwick, S.R., Pananos, A.D., Di Gregorio, S.E., Park, A.E., Etedali-Zadeh, P., Duennwald, M.L., Lajoie, P., 2016. A Toolbox for Rapid Quantitative Assessment of Chronological Lifespan and Survival in Saccharomyces cerevisiae. Traffic 17, 689–703. doi:10.1111/tra.12391

Structuring Experimental Data

The structure of the experimental data is extremely important. Experimental data should be saved as an .xlsx file (though the scripts can be easily changed so as to accommodate .csv files). The column headers should be the days the measurements were performed, while the rows should be the plasmid types. See Figure 1 for an example of the structure.

	A	B	C	D	E	F	G	H
1		Day 1	Day 2	Day 3	Day 6	Day 9	Day 14	Day 16
2	pBR1805 boi	251.61	247.84	252.32	250.24	236.381	176.118	175.157
3	PbR1805 uns	35.51	42.93	61.02	57.99	49.091	73.009	51.66
4	pBR1805 1	34.47	59.71	92.11	160.06	182.243	183.917	233.009
5	pBR1805 2	31.51	47.08	75.13	111.62	150.22	196.01	244.802
6	pBR1805 3	30.97	55.38	86.16	158.8	172.014	220.774	248.384
7	pBR1805 4	38.38	39.38	60.28	116.22	163.295	180.508	235.602
8	pBR1805 5	31.14	47.76	83.16	173.85	173.562	252.61	244.151
9	pBR1805 6	30.31	43.73	76.98	160.51	148.309	226.367	240.716

Figure 1: Note that the first row is the Positive Control measurements, the second row is the Negative Control measurements, followed by experimental measurements.

The `gen_data.py` Algorithms

The script `gen_data.py` takes as input experimental data formatted as in Figure 1, and will return .xlsx files of the survival rates and normalized survival rates to the working directory. The script will return data frames of the survival and normalized survival rates to the command line for further investigation if needed.

The script will splice out the positive and negative controls from the data, and will convert the experimental data to a matrix, $A_{n\times m}$. From here, a survival rate matrix, $B_{n\times m}$, is initialized, and each element is calculated using

$$b_{ij} = \begin{cases} 100\left(1 - \frac{a_{ij}}{a_{1j}}\right) & \text{if} \quad a_{ij} \leq a_{1j} \quad a_{1j} \neq 0 \\ 0 \quad \text{otherwise} \end{cases}$$

This will ensure that any negative survival rates due to loss of pigment will be measured as 0. Next, a normalized survival matrix, $C_{n\times m}$ is initialized. Elements of C are computed using

$$c_{ij} = 100\left(\frac{b_{ij}}{b_{i1}}\right) \quad b_{i1} \neq 0\,.$$

This expresses the survival rate as a percentage.

Calling `get_data()`

To use the script, call the function gen_data() from the python command line. First, ensure that your working directory is set to the location where the experimental data is saved. Use the following commands to generate the data:

```
from gen_data import *

sur, nor = gen_data( xlsx_file )
```

These commands will store the survival data frame in the variable `sur` and the normalized data frame as `nor`. The `.xlsx` files will be created and saved to the working directory.

If there is optical density data, then you may enter the keyword argument `od` followed by the optical density file name. Suppose I have optical density data in the file `optical_denisity.xlsx`. To compute survival and normalized survival data, enter the command:

```
sur, nor = gen_data(xlsx_file, od = 'optical_density.xlsx')
```

It is crucial you call the optical density file `od` and that its name is a string (i.e. surrounded in quotations).

Setting the `area` parameter to `True` will return the area under the Survival Rate curve. The algorithm calculates the area under the curve using the trapezoid rule for numerical integration with non-uniform grid spacing. For a data set containing *n* data points, the area is calculated using

$$\text{Area} = \frac{1}{2}\sum_{k=0}^{n-1}(t_{k+1} - t_k)(S_{k+1} - S_k)\ .$$

Here, t_k is the k^{th} time point, and S_k is the k^{th} survival data point.

Though the data can be plotted in graph pad, `plot_data.py` can be used to visualize the data for immediate feedback.

Calling `plot_data()`

Once the survival and normalized survival data frames have been created using the `get_data()` function, then either data frame can be plotted using `plot_data()`. To plot a data frame, use the following syntax:

```
from plot_data import *
```

```
plot_data(nor)
```

This command will plot the normalized survival rate data frame. Two plots are returned: A plot for each experiment over time, as well as the mean survival rates, complete with error bars using the standard error statistic.

A Complete Example

Suppose I have a data set called `my_experimental_data.xlsx`. I can create the survival and normalized survival rates excel files, as well as plot the data using the following syntax:

```
from gen_data import *

sur,nor = gen_data('my_experimental_data.xlsx', area =
True)

from plot_data import *

plot_data(nor)
```

Appendix B: Copyright information – Chapter 1

Copyright Statement

Appendix C: Copyright information – Chapter 2

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Publications:

Sarah R. Chadwick, Elena N. Fazio, Parnian Etedali-Zadeh, Julie Genereaux, Martin L. Duennwald, and Patrick Lajoie. 2019. A functional unfolded protein response is required for chronological aging in *Saccharomyces cerevisiae*. Current Genetics. DOI: 10.1007/s00294-019-01019-0

Sarah R. Chadwick and Patrick Lajoie. 2019. Endoplasmic reticulum stress coping mechanisms and lifespan regulation in health and diseases. Frontiers in Cell and Developmental Biology. DOI: 10.3389/fcell.2019.00084

Sarah R. Chadwick, A. Demetri Pananos, Sonja DiGregorio, Anna E. Park, Parnian Etedali-Zadeh, Martin L. Duennwald, and Patrick Lajoie. 2016. A toolbox of fluorescent assays to assess chronological lifespan in *Saccharomyces cerevisiae*. Traffic. DOI: 10.1111/tra.12391

Jiang Y.*, Sarah R. Chadwick* and Lajoie P. 2016. Endoplasmic reticulum stress – The cause and solution to Huntington's disease? Brain Research. DOI: 10.1016/j.brainres.2016.03.034 (* *indicates equal contribution*)

Selected Presentations:

Sarah R. Chadwick, Robyn D. Moir, Ian M. Willis, Erik L. Snapp, and Patrick Lajoie. Regulation of ER stress resistance by tauroursodeoxycholic acid in yeast. 2019. American Society for Cell Biology Annual Meeting, Washington, DC. (Poster presentation)

Sarah R. Chadwick. 2019. Regulation of ER stress sensitivity by tauroursodeoxycholic acid in yeast. Western Cell Stress Symposium, London, ON, Canada. (Research talk)

Sarah R. Chadwick and Patrick Lajoie. 2019. Investigating Tauroursodeoxycholic Acid's Role in Cell Stress Responses: Mechanistic Insights. PRinCE Conference 2019, Toronto, ON, Canada. (Poster presentation)

Sarah R. Chadwick**.** 2019. Stressed out: Examining endoplasmic reticulum stress responses and proteostasis in *Saccharomyces cerevisiae*. Anatomy and Cell Biology Seminar Series, London, ON, Canada. (Research talk)

Sarah R. Chadwick, Elena N. Fazio, Martin L. Duennwald and Patrick Lajoie. 2018. Multiple aspects of the unfolded protein response regulate chronological aging in *Saccharomyces cerevisiae*. London Health Research Day, London, ON, Canada. (Poster presentation)

Sarah R. Chadwick, Julie Genereaux, Parnian Etedali-Zadeh, Elena Fazio, Martin Duennwald, and Patrick Lajoie. 2017. Investigating the role of Inositol-Requiring Enzyme 1 (IRE1) in yeast Unfolded Protein Response (UPR), stress adaptation, and chronological aging. Anatomy and Cell Biology Research Day, London, ON, Canada. (Poster presentation)

Sarah R. Chadwick, Elena N. Fazio, Martin L. Duennwald and Patrick Lajoie. 2016. Assessing the role of endoplasmic reticulum stress signaling pathways in yeast chronological aging. American Society for Cell Biology Annual Meeting, San Francisco, CA. (Poster presentation)

Sarah R. Chadwick, A. Demetri Pananos, Sonja DiGregorio, Anna E. Park, Parnian Etedali-Zadeh, Martin L. Duennwald, and Patrick Lajoie. 2015. A toolbox of fluorescent assays to assess chronological lifespan in *Saccharomyces cerevisiae*. American Society for Cell Biology Annual Meeting. San Diego, CA. (Poster presentation)

Sarah R. Chadwick, Park E.J., Pananos AD., Martin L. Duennwald and Patrick Lajoie. 2015. Software-assisted assessment of chronological lifespan and survival using fluorescent assays in *Saccharomyces cerevisiae*. Anatomy and Cell Biology Research Day, London, ON, Canada. (Poster presentation)

Sarah Chadwick, Patrick Lajoie. 2015. Polyglutamine proteins and the unfolded protein response in aging. Departments of Biochemistry and Anatomy & Cell

Biology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, ON, Canada. (Poster presentation)

Conference Abstracts (* indicates presenting author):

Sarah R. Chadwick, A. Demetri Pananos, Sonja DiGregorio, Eun J. Park, Parnian Etedali-Zadeh, Martin L. Duennwald and Patrick Lajoie*. 2016. Assessing the role of endoplasmic stress signaling pathways in yeast chronological aging. North East Regional Yeast Meeting. Buffalo, NY USA

Jiang Y., Fazio EN., Chadwick SR., Duennwald ML. and Lajoie P*. 2015. Mechanisms of stress tolerance in a yeast model of Huntington's disease. International Yeast Meeting, Levico Terme, Italy

Fazio EN., Jiang Y., Chadwick SR., Duennwald ML. and Lajoie P*. 2015. The impact of expanded polyQ proteins on the endoplasmic reticulum homeostasis during aging: lessons from a yeast model of Huntington's disease. FASEB summer conference: From unfolded proteins in the endoplasmic reticulum to diseases. Saxton River, VT USA