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RNA Regulation During the Unfolded Protein Response in Yeast

by

Marcy Ann Diaz

DISSERTATION

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Abstract

In eukaryotic cells, all secreted and transmembrane proteins are trafficked through the endoplasmic reticulum (ER). They are accurately translated, folded and modified within the specialized environment of the ER. The fidelity of protein processing within the ER is crucial for cell survival. Therefore, mechanisms have evolved that regulate translation, translocation and proper processing of newly synthesized proteins. The unfolded protein response (UPR) is one such pathway that monitors the protein folding capacity of the ER. In all organisms analyzed to date, the UPR drives transcriptional programs that allow cells to cope with ER stress. In budding yeast, the key regulatory step in this pathway is the non non-conventional splicing of HAC1 encoding a transcriptional activator. This splicing event is conserved in higher organisms and dependent on Ire1, an ER membrane-resident kinase/endoribonuclease. This thesis adds to the continuing story of the UPR by extending the study of this conserved pathway into the fission yeast Schizosaccharomyces pombe. We found that the fission yeast S. pombe lacks both a Hac1 ortholog and a UPR-dependent transcriptional program. Instead, Ire1 initiates the selective decay of a subset of ER-localized mRNAs that is required to survive ER stress. We identified Bip1 mRNA, encoding a major ER chaperone, as the sole mRNA cleaved upon Ire1 activation that escapes decay. Instead, truncation of its 3’ UTR, including loss of its polyA tail, stabilized Bip1 mRNA, resulting in increased BiP translation. Thus, S. pombe uses a universally conserved stress-sensing machinery in novel ways to maintain homeostasis in the ER. Additionally, we present data that shows that mutations in the RNA degradation pathway in budding yeast are able to suppress the UPR specific growth defect of a mutant allele of tRNA ligase, trl1-100. We also studied
the regulation of translation by genome wide high throughput ribosome footprinting in cells with harboring mutated ribosomes and cells lacking a key component of the machinery that targets proteins for co-translational translocation into the ER. Together the work presented here attempts to understand the role that mRNA regulation plays in various mechanisms regulating protein synthesis, translocation and ER homeostasis.
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Chapter 1

Introduction: The Role of RNA Regulation in the Unfolded Protein Response
All proteins must be accurately translated, folded, modified and transported in order to carry out their proper function in the appropriate location. Secreted and transmembrane proteins require special consideration because they are essential for all cells to successfully communicate with their environment. Failure to properly translate, fold, or localize these proteins can result in catastrophic consequences for the cell. Therefore, mechanisms have evolved to regulate translation, optimize protein folding, and ensure the quality of newly synthesized proteins. These complex regulatory processes all utilize the regulation of mRNA abundance or mRNA translation through unique mechanisms to ensure the fidelity of secreted and transmembrane proteins. This thesis will examine the regulatory role that RNA plays in the Unfolded Protein Response in budding and fission yeast.

The ER and Protein Folding

The ER is a membranous organelle that serves as the entry point for the secretory pathway and the primary site of most lipid biosynthesis. The structure, shape, and appearance of the ER are all integrally related to its function. For example, the smooth ER provides an ideal location for lipid biosynthesis, while the rough ER is covered in ribosomes and is the site of co-translational protein translocation into the ER. In eukaryotic cells, nascent proteins destined for membrane insertion or secretion are translocated as unfolded polypeptides into the endoplasmic reticulum (ER). The lumen of the ER provides an oxidizing environment ideal for protein folding and modification. The lumen is filled with enzymes to facilitate protein folding, protein-processing enzymes for glycosylation and disulfide bond formation and ER chaperones.
Most proteins that enter the secretory pathways are targeted to the ER via an N-terminal hydrophobic signal sequence. The Signal Recognition Particle (SRP) recognizes and binds to this sequence as the nascent protein emerges from the ribosome, stalling translation (Walter and Blobel, 1981). The SRP then targets the signal peptide and associated ribosome to the SRP receptor on the cytosolic surface of the ER (Gilmore et al., 1982a; Gilmore et al., 1982b), where the complex is transferred to the translocon. Once bound to the translocon, the ribosome continues translation of the protein across the ER membrane. Co-translational targeting of membrane bound and secreted proteins ensures that proteins do not aggregate or fold prior to entering the specialized environment of the ER.

At times throughout the lifecycle of the cell, the cell encounters physiological conditions that impair the secretory pathway or increase the number of secreted proteins. At these times, the protein folding demand may exceed the folding capacity of the ER. The ER is a dynamic organelle that is constantly remodeling and expanding to meet the demands of each individual cell. Conditions that increase the amount of unfolded protein within the lumen of the ER are broadly characterized as “ER stress”. The cell has evolved a mechanism to cope with such changing demands upon the ER, termed the Unfolded Protein Response (UPR). The UPR is an ER to nucleus signal transduction pathway that monitors “ER stress” and, according to need, tunes the expression of genes involved in protein folding, lipid biosynthesis and protein secretion, among others (Travers et al., 2000). Thus, the UPR maintains protein homeostasis and allows the cell to overcome ER stress.
Conceptually, the UPR utilizes two distinct strategies to restore protein homeostasis upon ER stress; it can increase the folding capacity of the ER or it can decrease the folding load upon the ER. The UPR was initially characterized as a vast transcriptional program that upregulates folding machinery and lipid biosynthesis machinery, among others, in order to increase the size and folding capacity of the ER (Travers et al., 2000). This function has been conserved from fungi to metazoans, and further elaborated upon with additional branches to the UPR activating different transcriptional factors. However, more recently the UPR in metazoans has been shown to function through translational repression and ER localized mRNA degradation to decrease the folding load in the ER (Harding et al., 1999; Hollien et al., 2009; Hollien and Weissman, 2006). Therefore, in metazoans, the UPR not only activates a transcriptional response to increase protein folding capacity, but also additionally functions to decrease the protein folding load.

Malfunction of the UPR, either through the inability to restore protein folding homeostasis or from an overactive UPR, can have drastic consequences for the cell. This is underscored by the role the UPR plays in the progression of many diseases, especially those of dedicated secretory cells, including diabetes, multiple myeloma and neurodegenerative diseases (Lin et al., 2008). The cytoprotective functions of UPR signaling are mis-appropriated in a variety of cancer cells. Prolonged activation of the UPR, however, can lead to apoptosis (Lin et al., 2007; Ma and Hendershot, 2004). Understanding the regulation of the UPR will help us utilize both the cytoprotective and cytotoxic possibilities of the UPR for therapeutic advantage.
**General UPR in *S. cerevisiae***

The UPR was originally discovered in the yeast *Saccharomyces cerevisiae*. In this organism, three factors mediate the UPR: Ire1, Trl1, and Hac1. Ire1 is a transmembrane kinase and endonuclease that acts as a signal from the ER to the nucleus (Cox et al., 1993; Mori et al., 1993). Trl1 is the yeast tRNA ligase and is essential for completing the signal originating from Ire1 (Sidrauski et al., 1996). Hac1 is a bZIP transcription factor responsible for the activation of genes that increase the capacity of the ER (Cox and Walter, 1996; Mori et al., 1996). Together these components create an efficient signaling mechanism, outlined in Figure 1, for sensing the protein folding needs within the lumen of the ER, transmit that signal to the nucleus and upregulate a vast transcriptional response (Travers et al., 2000).

When misfolded proteins accumulate within the lumen of the ER, Ire1, a transmembrane protein consisting of a luminal sensing domain and cytoplasmic effector domains, is responsible for sensing ER stress and initiating the UPR (Cox et al., 1993; Mori et al., 1993). Upon detection of ER stress, likely via direct binding of the luminal domain to unfolded proteins (Credle et al., 2005; Gardner and Walter, 2011), Ire1 oligomerizes. Oligomerization brings the cytoplasmic domains, a kinase and an RNase, into close proximity, initiating trans-autophosphorylation and activation of the RNase domains (Shamu and Walter, 1996). While phosphorylation cascades are a common mechanism for signaling pathways, the activation cascade of Ire1 is very unique. Signal transmission relies not on phosphorylation, but on a non-conventional cytoplasmic splicing of the *HAC1* mRNA (Cox and Walter, 1996; Rüegsegger et al., 2001). The
RNase domain is allosterically activated by nucleotide binding in the kinase, but does not actually require phosphorylation of Ire1.

Under non-stress conditions, the mRNA encoding for Hac1 resides in the cytoplasm in a translationally silent state. The \textit{HAC1} mRNA contains a non-spliceosomal regulatory intron that blocks protein production to ensure that no Hac1 protein is made in non-stress conditions (Rüegsegger et al., 2001). The activated Ire1 RNase domain cleaves the \textit{HAC1} mRNA at two conserved stem loops flanking the \textit{HAC1} intron (Gonzalez et al., 1999). Trl1 ligates the exons together to form the translationally competent \textit{HAC1} mRNA (Sidrauski et al., 1996). The \textit{HAC1} mRNA is then free to be translated into the transcriptional activator Hac1. Newly synthesized Hac1 travels to the nucleus to upregulate the genes involved in the UPR. Thus, the key regulatory step in the UPR is the unique cytoplasmic splicing of the \textit{HAC1} mRNA by Ire1.

The unconventional splicing of \textit{HAC1} functions as a switch between two forms of the \textit{HAC1} mRNA, the unspliced mRNA, termed \textit{HAC1}^u, and the spliced mRNA, called \textit{HAC1}^i. This splicing relieves the translational attenuation of the \textit{HAC1} mRNA through the removal of an inhibitory intron (Rüegsegger et al., 2001). This feature of the UPR can function as a regulatory element because under non-stress conditions the \textit{HAC1}^u mRNA is translationally silent. \textit{HAC1}^u is an abundant cytoplasmic mRNA. However, no protein is detected in non-UPR conditions. This translational repression is dependent on a 16 nucleotide basepairing interaction between the 5’ untranslated region (UTR) and the intron of the message. When this interaction is relieved through mutation or the binding of a complementary oligonucleotide, the Hac1 protein is made (Rüegsegger et al., 2001). Despite this inhibitory interaction, evidence suggests that \textit{HAC1}^u is loaded with
ribosomes indicating that ribosomes are able to bypass the basepairing interaction and are regulated at the level of elongation (Chapman and Walter, 1997; Rüegsegger et al., 2001). Three possibilities exist: ribosomes are loaded during translocation from the nucleus into the cytoplasm before basepairing is fully achieved and basepairing leads to a block in initiation and elongation; basepairing breathes and lets ribosomes load onto the mRNA; or basepairing acts as an IRES allowing ribosomes to load onto the mRNA freely.

While the key regulatory step of the budding yeast UPR is the unique cytoplasmic splicing carried out by the endoribonucleolytic activity of Ire1, another important regulatory element in this splicing reaction is the yeast tRNA ligase, Trl1. tRNA ligase is an essential protein in budding yeast. Surprisingly, Trl1 was also found to be required for cell survival upon the induction of ER stress (Sidrauski et al., 1996). A genetic screen to identify essential components of the UPR identified a single defective allele of TRL1, called trl1-100. Upon UPR induction, cells harboring the trl1-100 allele of tRNA ligase are unable to ligate the ends of the HAC1 mRNA in vivo, yet the enzyme retains its ability to participate in tRNA ligation (Sidrauski et al., 1996). However, purified trl1-100 is able to splice HAC1 in vitro (Gonzalez, 2003). The mechanism of HAC1 splicing was found to be comparable to the mechanism by which tRNAs are spliced (Gonzalez et al., 1999). Both tRNA endonuclease and Ire1 generate 2’,3’-cyclic phosphates when they cleave their substrate RNAs, yielding similar substrates for Trl1 to recognize. Both processes use the same mechanism and enzyme for ligation, however HAC1 splicing occurs in the cytoplasm (Rüegsegger et al., 2001), whereas tRNA splicing is thought to occur in the nucleus (Clark and Abelson, 1987). Together, this evidence presents several
questions: how does Trl1 coordinate the splicing of tRNAs in the nucleus and get recruited to centers of \(HAC1\) mRNA splicing? How does the trl1-100 mutation uncouple \(HAC1\) splicing from tRNA splicing when it presumably utilizes the same mechanism for both processes?

The UPR in Higher Organisms

While the UPR in \(S.\ cerevisiae\), as we currently understand it, is a transcriptional response, the UPR in metazoans is much more complex. Three separate ER resident transmembrane proteins have evolved: Ire1 (inositol requiring enzyme 1), ATF6 (activating transcription factor 1) and PERK (PKR-like ER kinase). Together, these proteins initiate a UPR that utilizes both strategies for restoring ER homeostasis; they increase protein folding capacity and decrease protein folding load. The Ire1 and ATF6 branches mediate a transcriptional response, similar to that of \(S.\ cerevisiae\), that upregulate genes to expand the capacity of the ER. PERK, however, reduces global translation to slow protein production and thus influx of proteins into the ER (Pavitt and Ron, 2012). Additionally, Ire1 not only cleaves the mRNA of a dedicated transcription factor, but also downregulates mRNAs coding for actively translocating proteins in a process termed regulated Ire1-dependent decay (RIDD) (Hollien et al., 2009; Hollien and Weissman, 2006). All mRNAs downregulated in an Ire1 dependent fashion are cotranslationally targeted to the ER membrane. It is believed that Ire1 is directly cleaving these mRNAs upon sensing of ER stress (Hollien et al., 2009). These cleaved mRNAs are not spliced by tRNA ligase, but are subject to rapid decay by the cell’s RNA degradation pathways (Garneau et al., 2007), therefore reducing protein load in the ER.
In eukaryotic cells, mRNA is degraded by two major pathways, either from the 5’-3’ or the 3’-5’ direction. Both pathways are initiated by the loss of the polyadenylated (polyA) tail of an mRNA (Coller and Parker, 2004). In addition to specific deadenylases that shorten the polyA tail, regulated endonucleolytic cleavage can also initiate RNA degradation, as has been observed with Ire1 (Hollien et al., 2009; Hollien and Weissman, 2006; Sidrauski et al., 1996), or through special quality control mechanisms that monitor mRNAs for the presence of a premature stop codon or the absence of a stop codon (Frischmeyer et al., 2002; Muhlrad and Parker, 1994; van Hoof et al., 2002). Most often the shortening of the polyA tail is followed by decapping of the mRNA and the mRNA is then degraded by the 5’-3’ exoribonuclease Xrn1. However, mRNA can also be degraded in the 3’-5’ direction in a process initiated by mRNA association with cytosolic complexes such as the Ski2/3/8 complex and Ski7 in the cytoplasm (Anderson and Parker, 1998) (Araki et al., 2001) and the TRAMP complex in the nucleus (LaCava et al., 2005; Vanacova et al., 2005; Wyers et al., 2005). The RNA exosome, a multi-subunit complex with 3’ to 5’ exoribonuclease activity, then degrades the mRNA (Mitchell et al., 1997).

The UPR in the Fission Yeast S. pombe

Prior to the work presented in this thesis, the UPR in the fission yeast S. pombe was largely unexplored. It wasn’t until recently that bioinformatic analysis done in our lab identified that, while Ire1 is conserved through all yeast species, members in the genus Schizosaccharomyces do not have a homolog of the Hac1 transcription factor. Fission yeast have the machinery to sense unfolded proteins, an Ire1 homolog, but do not
have the transcription factor. This poses the immediate question: are these cells responding to ER stress and, if so, how? To date, the most evolutionarily basic UPR has been defined as the transcriptional response mediated by activation of Ire1 found in *S. cerevisiae*. However, the study of Ire1 in fission yeast offers a new perspective on the UPR and its evolutionary basis.

The work presented in this thesis broadly encompasses elements of RNA regulation that are required during the UPR to ensure ER homeostasis and the regulation of ribosomes during translation and translocation into the ER. Chapter 2 discusses the UPR in *S. pombe* and the identification of the first UPR that does not utilize a transcriptional response. This work bridges an evolutionary gap between the UPR mechanisms of *S. cerevisiae* and the more complex UPR found in mammalian cells. By studying the UPR in the fission yeast *S. pombe*, we have discovered a different strategy for re-establishing homeostasis after protein folding stress. Chapter 3 describes preliminary efforts to further characterize *S. pombe* Ire1. In Chapter 4 we outline continued work to identify dominant suppressors of the *trl1*-*100* mutation and identify a mutation in the *SKI2* gene. In Chapter 5 we discuss using genome wide ribosome mapping to look at global translational regulation in cells harboring ribosomes with mutated exit tunnels or lacking the SRP receptor.
Ire1 resides in the ER membrane and is activated when it senses ER stress in the form of direct binding to misfolded proteins within the lumen of the ER. Upon activation, Ire1 molecules oligomerize, which activates the cytoplasmic endonucleolytic activity of the enzyme. Activated Ire1 then cleaves its substrate, the HAC1 mRNA, in the cytoplasm. This action removes an inhibitory intron from the mRNA and the exons are ligated by tRNA ligase. The spliced form of HAC1 is then rapidly translated into the Hac1 transcription factor. The transcription factor then travels to the nucleus where it upregulates UPR target genes.
Figure 1.
References

Anderson, J.S., and Parker, R.P. (1998). The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex. EMBO J 17, 1497-1506.


Chapter 2

The Unfolded Protein Response in Fission Yeast Modulates Stability of Select mRNAs to Maintain Protein Homeostasis
The Unfolded Protein Response in Fission Yeast Modulates Stability of Select mRNAs to Maintain Protein Homeostasis

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Abstract

The unfolded protein response (UPR) monitors the protein folding capacity of the endoplasmic reticulum (ER). In all organisms analyzed to date, the UPR drives transcriptional programs that allow cells to cope with ER stress. The non-conventional splicing of *Hac1* (yeasts) and *XBP1* (metazoans) mRNA, encoding orthologous UPR transcription activators, is conserved and dependent on Ire1, an ER membrane-resident kinase/endoribonuclease. We found that the fission yeast *Schizosaccharomyces pombe* lacks both a Hac1/XBP1 ortholog and a UPR-dependent transcriptional program. Instead, Ire1 initiates the selective decay of a subset of ER-localized mRNAs that is required to survive ER stress. We identified *Bip1* mRNA, encoding a major ER chaperone, as the sole mRNA cleaved upon Ire1 activation that escapes decay. Instead, truncation of its 3’ UTR, including loss of its polyA tail, stabilized *Bip1* mRNA, resulting in increased BiP translation. Thus, *S. pombe* uses a universally conserved stress-sensing machinery in novel ways to maintain homeostasis in the ER.
Introduction

Homeostatic control mechanisms are essential to life, allowing cells to balance capacity and demand of numerous physiological processes. One such mechanism, the unfolded protein response (UPR), operates in all eukaryotic cells to adjust the protein folding capacity of the endoplasmic reticulum (ER) according to need. Environmental or physiological demands can lead to an imbalance between the protein folding load and the protein folding capacity in the ER lumen, resulting in an accumulation of unfolded or misfolded proteins, a condition termed “ER stress” (1). When unmitigated, ER stress is toxic to cells and triggers cell death (2-4).

The UPR is a network of evolutionarily conserved signal transduction pathways that monitors the conditions in the ER lumen to induce a transcriptional response. In metazoan cells, three ER-resident transmembrane sensors, Ire1, PERK, ATF6 transmit information into the cytosol. Each sensor activates transcription factors that collaborate to drive expression of UPR target genes (1), including genes encoding ER-lumenal chaperones, such as BiP, an abundantly expressed Hsp70 family member.

Ire1 is a bifunctional transmembrane kinase/endoribonuclease that controls expression of the transcription factor XBP1 by a non-conventional splicing of its mRNA. Ire1 uses its ER-lumenal domain to detect unfolded proteins, and in response activates by homo- oligomerization, trans-autophosphorylation, and allosteric activation of its cytosolic nuclease modality (5, 6). Activated Ire1 cleaves the XBP1 mRNA at two discrete stem-loop structures, excising a short intron. The two severed exons are then ligated to produce spliced XBP1 mRNA, which because of a frame-shift induced by the splicing event, are translated to produce active XBP1 (7, 8).
Ire1 was first discovered in the budding yeast *S. cerevisiae*, where it constitutes the core machinery of the cells’ only UPR signaling pathway (9, 10). *S. cerevisiae* Ire1 splices *Hac1* mRNA, encoding the yeast ortholog of XBP1, by a mechanism that was later found conserved in all metazoan cells (11, 12). Ire1-mediated mRNA splicing therefore is considered to be the most evolutionary ancient branch of the UPR.

By first approximation, the three UPR branches collaborate to effect comprehensive transcriptional outputs, thereby enhancing the capacity of the ER according to need. PERK superimposes another layer of control by reducing the load of proteins entering the ER through translational control (13). Similarly, Ire1 is thought to play a dual role in UPR regulation. In particular, Hollien and Weissman first discovered in *Drosophila* cells that Ire1 induction not only results in splicing of *XBP1* mRNA but also mediates enhanced mRNA breakdown (14). This output of Ire1 activation, termed “regulated Ire1-dependent decay” (RIDD), is conserved in mammalian cells, but not in *S. cerevisiae*, where transcriptional control via *Hac1* mRNA splicing remains the only known route of UPR signaling (15-17). All identified RIDD target mRNAs are translated by membrane-bound ribosomes at the ER surface, where they are cleaved, most likely by Ire1 directly (15, 16, 18). Once nicked and no longer protected by their polyA tails and 5’ caps, mRNA fragments are quickly degraded by the RNA surveillance machinery (14, 19).

By contrast to the strictly conserved stem/loop structures found at *Hac1/XBP1* mRNA splice sites (20), RIDD target mRNAs do not contain easily recognizable features in common. Consequently, RIDD is thought to arise by a more promiscuous cleavage mode of Ire1. It is unclear whether RIDD is mediated by an alternate conformation of
activated Ire1, or whether it arises in a specific Ire1 oligomerization state, as high-order oligomerization may serve to locally enhance low affinity interactions through avidity effects. RIDD cleavage reactions have been reconstituted in vitro with recombinantly expressed purified Ire1, lending support to the notion that Ire1’s endoribonuclease activity, rather than another enzyme recruited to it, carries out the initial cleavage reaction (18, 21).

Because of Ire1’s dual output, the physiological consequences of RIDD have been difficult to decipher. RIDD has been suggested to play cytoprotective roles, such as contributing to important feedback control on proinsulin expression in pancreatic beta-cells or protecting liver cells from acetaminophen toxicity by degrading the mRNAs encoding the cytochrome P450 variants responsible for the drug’s toxification (22, 23). RIDD has also been suggested to play cytotoxic roles as a major contributor driving cells into apoptosis after prolonged and unmitigated exposure to ER stress (16).

Surprisingly, in the work presented here we found no evidence that Ire1 controls transcription in the UPR of Schizosaccharomyces pombe. Instead, in S. pombe Ire1 maintains ER homeostasis through two post-transcriptional mechanisms: it initiates RIDD of a large, select set of ER-targeted mRNAs and processes Bip1 mRNA in an unprecedented way, thereby stabilizing it. Our studies reveal an unforeseen evolutionary plasticity in maintaining ER homeostasis.
Results

A Functional Unfolded Protein Response in Fission Yeast

UPR induction in all eukaryotic cells analyzed to date involves the Ire1-mediated, non-conventional splicing of Hac1/XBP1 mRNA. The splice sites at which Ire1 cleaves the mRNA to initiate splicing lie in well-conserved stem/loop structures that are readily identified (20). We and others were therefore perplexed when bioinformatic analyses failed to identify Hac1/XBP1 orthologs in S. pombe and other yeasts of the same genus (Figure 1a) (24, 25). The Hac1/XBP1 transcription factors are well conserved between species and are easily recognized by sequence alignment among the superfamily of bZIP transcription factors (Figure 1 - S1). By contrast, Ire1 is well conserved in S. pombe, with all of the functionally important hallmarks identified in other eukaryotes, including its ER lumenal unfolded protein sensing domain and its cytosolic kinase and RNase domains. Moreover, Ire1 was essential for S. pombe growth on tunicamycin (Tm) (Figure 1b), which induces ER stress by blocking N-linked glycosylation, indicating that Ire1 serves an essential function in allowing cells to cope with ER stress. This function required Ire1’s RNase activity, as Ire1(H1018N) carrying a single amino acid substitution of a catalytic residue in Ire1’s RNase active site failed to support cell growth on tunicamycin (Figure 1b).

ER Stress Dependent mRNA Down-Regulation

To address the conundrum posed by the missing Ire1 splicing substrate in S. pombe, we first explored the scope of UPR-dependent changes in gene expression. To this end, we isolated polyA⁺ RNA from wild type and Ire1Δ cells, in which the UPR was
induced with the reducing agent dithiothreitol (DTT). DTT causes ER stress by impairing disulfide bond formation in the ER. The purified mRNA population was reverse-transcribed and subjected to deep-sequencing. Unexpectedly, we observed widespread Ire1-dependent mRNA down-regulation, but virtually no mRNA up-regulation (Figure 1c). Thirty-nine mRNA species were reduced by more than two-fold in a DTT- and Ire1-dependent manner (Figure 1c, bottom left grayed area). Most members of this set of down-regulated mRNAs were abundantly expressed, as depicted by the size of the plotted circles. Down-regulation, however, did not correlate with mRNA abundance (Figure 1 – S2). Intriguingly, the set of down-regulated genes exclusively encoded proteins targeted to the ER (identified by signal sequences and/or transmembrane segments) (Figure 1c, red circles). As shown in Figure 1d, the genome-wide profile of Ire1- and ER stress-dependent mRNA changes of genes encoding ER-targeted proteins is skewed to a significantly greater extent towards down-regulation than that of other mRNAs ($P < 1 \times 10^{-20}$). More than half of the most down-regulated mRNAs encode proteins with annotated functions in the secretory pathway, in particular proteins involved in lipid metabolism, trafficking, and ER functions (Figure 1e).

As the reduction in mRNA abundance was ER stress- and Ire1-dependent, we next explored if Ire1 could be directly involved in destabilizing ER-bound mRNAs. To this end, we sought to trap any putative primary Ire1-cleavage products prior to degradation by deleting $Ski2$, which encodes a helicase component of the cytosolic Ski complex (cytosolic exosome) that mediates 3’ $\rightarrow$ 5’ RNA decay. Northern blot analysis of $Ski2\Delta$ cells revealed that $Gas2$ mRNA (which is down-regulated 2.5-fold in an ER stress and Ire1-dependent manner) yielded two discrete cleavage products upon ER stress

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Gas2 mRNA cleavage was dependent on Ire1, as no mRNA reduction and no cleavage products were observed in *Ire1Δ Ski2Δ* double deletion cells (Figure 2a). Another target, *Yop1*, behaved similarly (Figure 2 - S1). In time-course experiments, reduction of *Gas2* mRNA and accumulation of the cleavage products peaked at 30 min after UPR induction (Figure 2b); at later time points the abundance of intact full-length mRNA increased, suggesting that newly transcribed mRNA is not cleaved if the Ire1-dependent cleavage products are not further degraded. Indeed, *Ski2Δ* cells failed to grow on plates containing tunicamycin (Figure 2c), indicating that an intact mRNA decay is important for *S. pombe* cells to cope with ER stress.

To determine the mRNA cleavage sites genome-wide, we prepared total RNA fractions from *Ski2Δ* and from *Ire1Δ Ski2Δ* cells. We used tRNA ligase to attach linker sequences specifically to those RNA fragments terminating in a 2′,3′-cyclic phosphate, which is the expected product of Ire1-catalyzed RNA cleavage (26). We then amplified the cleavage products in 3’ RACE reactions priming at the linker sequence. Alignment of the sequencing data to the *S. pombe* genome identified the 3’ ends of Ire1-dependent fragments. In particular, we identified 39 Ire1-dependent fragments mapping to 24 of the most down-regulated genes, as shown in Figure 2d for *Gas2* mRNA (left panel). By size estimation, the major Ire1-dependent peak corresponded to the smaller, more abundant *Gas2* mRNA cleavage product (labeled ▲ in Figs. 2a and 2b). A second, less abundant short fragment was also observed in the sequencing data (labeled ✗ in Figure 2d). (Fragment ✗ was absent or below the detection limit on the Northern blot unless the primary cleavage site was mutated (see Figure 2g, discussed below).) Spliceosomal U6
RNA normally terminates in a 2',3'-cyclic phosphate and thus provided a valuable control for the ligation reaction (Figure 2d, right panel).

Alignment of the experimentally determined Ire1-dependent cleavage sites revealed a core motif with a signature of three conserved nucleotides (UGiC) that flank the Ire1-dependent cleavage sites at positions -2, -1, and +1 with an additional strong bias against G in position +2 (Figure 2e). Most mapped mRNA cleavage sites (34 of 39), including those in Gas2 mRNA, localized within the open reading frames. Indeed, a Gas2 reporter construct transcribed off a heterologous alpha-tubulin (Nda2) promoter and containing only the Gas2 ORF flanked by heterologous 5' and 3' tubulin untranslated regions (UTRs), was down-regulated upon ER stress in an Ire1-dependent manner (Figure 2f). This degradation was quantitatively comparable to that of the native Gas2 transcript (Figure 2 - S2), indicating that the information contained within the Gas2 ORF is sufficient to confer susceptibility to Ire1-dependent cleavage.

To assess the functional importance of the identified Gas2 mRNA cleavage site experimentally, we mutated the UGC-residues of the ▲-site (UGiCU). As expected, ER stress-dependent cleavage of the Gas2 reporter mRNA at the mapped site was abolished (Figure 2g). In its place, however, we observed two new Ire1-dependent fragments (labeled × and †). Scanning gel densitometry revealed that fragment × is distinctly smaller than fragment ▲, and hence represents a cryptic site that is only utilized when site ▲ is mutated. Fragment † likely corresponds to the lower abundance cleavage product observed in Figures 2a and 2b, which becomes more prominent in the mutant construct. Taken together, we conclude that Ire1-dependent mRNA cleavage in S. pombe is sequence dependent.
The data presented so far suggest that homeostatic control of ER protein folding is regulated differently in *S. pombe* than *S. cerevisiae*. Rather than relying on a transcriptional program to upregulate genes that enhance ER protein folding capacity as in *S. cerevisiae*, *S. pombe* cells reduce the amount of specific proteins entering the organelle by decreasing the level of ER-targeted mRNAs using Ire1-dependent mRNA degradation.

**Bip1 mRNA Processing and Stabilization in Response to ER stress**

In all species analyzed to date, Bip1 is a major UPR target gene that is upregulated when cells experience ER stress. Paradoxically, we found *S. pombe* Bip1 mRNA among the 39 down-regulated mRNAs identified by the analyses shown in Figure 1c. Analysis by Northern blotting yielded seemingly conflicting results: by this analysis, Bip1 mRNA was 4-fold more abundant in ER stressed cells (Figure 3a). Intriguingly, the appearance of a faster migrating mRNA species ("tBip1 mRNA") indicates that Bip1 mRNA changes size in cells experiencing ER stress (Figure 3a, lanes 3-4). Appearance of the tBip1 mRNA species was Ire1-dependent and in wild type cells accounted for the increase in overall mRNA abundance. The increase did not result from augmented transcription. We measured the activity of a heterologous reporter in which the Bip1 promoter was fused to GFP and showed no Ire1-dependent change in mRNA abundance with ER stress (Figure 3b). In agreement with this result, we found that the stability of an mRNA bearing the Bip1 ORF and 3’ UTR showed a more than 3-fold increase in half-life from $T_{1/2} = 20$ min for the unprocessed form present in unstressed cells to $T_{1/2} = 70$ min for the processed form present in ER-stressed cells (Figure 3c). Furthermore, the Bip1 3’
UTR and the presence of a signal sequence were sufficient to a heterologous mRNA construct to confer Ire1-dependent processing (Figure 3 – S1).

Sequencing of the expressed genome in UPR-induced and uninduced cells revealed the molecular difference between Bip1 and tBip1 mRNA (Figure 3d). For these experiments, we extracted total RNA and then, without selecting for polyA+ RNA, removed rRNA by subtractive hybridization. After reverse transcription, deep-sequencing of the cDNA pool from uninduced cells revealed good coverage of reads spanning the entire Bip1 mRNA including its 5’ and 3’ UTR (Figure 3d, left, blue profile). By contrast, cDNA isolated from DTT-treated cells revealed a precipitous drop in reads mapping to the 3’ end (Figure 3d, left, red profile and Figure 3 - S2). We also performed 3’-RACE to determine the 3’ end of tBip1 mRNA. The sequence of the amplified DNA confirmed that tBip1 mRNA lacks a polyA tail and terminates at G373 in the 3’ UTR (Figure 3d, right panel). In 7 independently isolated clones, we found no sequence variations in the tBip1 linker junction. The sequences flanking G373 align with the UG\CU motif (Figure 2e), suggesting that tBip1 mRNA is produced by truncation of Bip1 mRNA in an Ire1-dependent RNA cleavage reaction that resembles those of the Ire1-dependently down-regulated mRNAs described above.

Mutational analysis of the cleavage site confirmed that specific sequences are required. Mutation of G373 to C or U and its deletion together with preceding nucleotides abolished Ire1-dependent Bip1 mRNA processing (Figure 3f). By contrast, a mutation of the preceding G370 to C diminished cleavage only marginally (<2-fold). In all analyzed mutants of Bip1 mRNA, UPR-induction increased abundance of the transcript ~2-fold (a level comparable to that observed in Ire1Δ cells) (Figure 3a, right
panel), whether processing took place or not, perhaps due to compensatory transcriptional regulation that is independent of Ire1. For all mutants, however, the increased abundance stayed shy of the 4-fold increase observed for wild type Bip1 mRNA.

As Bip1 mRNA truncation resulted in a loss of the polyA tail, this result resolves the paradox of why Bip1 mRNA appeared to be down-regulated in the polyA+ mRNA pool analyzed in Figure 1c. Indeed, directly comparing the UPR-dependent fold-change in mRNA abundance of polyA+ RNA and rRNA-depleted total RNA uniquely positioned Bip1 sequences as an anti-correlated outlier, whereas all other mRNAs were well correlated between the samples (Figure 3e). From these data we conclude that, remarkably, Bip1 mRNA is the only stable mRNA in the cell that loses its polyA tail upon UPR induction.

It was unexpected to find an mRNA that had lost its polyA tail to be more stable in cells. To determine the translation proficiency of tBip1 mRNA, we subjected UPR-induced cells to polysome profiling. These experiments confirmed that despite lacking its polyA tail, tBip1 mRNA sedimented in the polyribosome fractions in sucrose gradients (Figure 4a). Moreover, ribosome footprinting demonstrated that Bip1 mRNA in uninduced cells and tBip1 mRNA in UPR-induced cells were engaged with actively translating ribosomes, mapping throughout the Bip1 ORF (Figure 4b). The larger number of reads obtained upon UPR induction correlated with the higher abundance of tBip1 mRNA. We note a significant ribosome occupancy preceding the translation start site in both Bip1 and tBip1 mRNA most likely presenting previously unrecognized small uORFs (see Figure 4 – S1 for a zoomed-in view). The relative ribosome occupancy of these putative uORFs did not change with UPR induction. Translation of the processed mRNA
resulted in an enhanced steady-state concentration of Bip1 protein, as shown by quantitative Western blotting (Figure 4 – S2).

To assess the physiological consequences of this unique regulatory mechanism of Bip1 expression, we explored the growth of strains carrying a mutation of the Bip1 mRNA processing site (ΔTTAACTGGTG/C). Liquid cultures of Bip1 mRNA mutant, that were exposed to a pulse of ER stress (tunicamycin) and allowed to recover after washout of the drug showed a marked growth delay in early log phase (Figure 4c) and enhanced cell death (Figure 4d), indicating that Bip1 mRNA processing is important for maintaining cell fitness in the face of ER stress. By contrast to cell growth in liquid culture, Bip1 mutant cells grew on UPR-inducing tunicamycin plates only marginally worse that wild type cells (Figure 4 – S3). The importance of Bip1 mRNA processing, therefore, varies with growth conditions.

**Discussion**

We have begun to characterize the UPR in fission yeast. To our surprise, we discovered that—by contrast to all other eukaryotes studied to date—*S. pombe* does not utilize Ire1, the most ancient ER stress sensor, to control transcription. Rather, *S. pombe* exclusively relies on two means of Ire1-dependent post-transcriptional regulation to cope with ER stress:

i) Regulated Ire1-dependent mRNA decay (RIDD) of a large and highly select set of mRNAs, all of which are predicted to be translated by membrane-bound ribosomes at the ER. The mechanistic features of mRNA degradation in *S. pombe* are highly
reminiscent of RIDD previously described in insect and mammalian cells, where it accompanies Ire1-dependent mRNA splicing.

ii) Processing of Bip1 mRNA within its 3’ UTR, leading to loss of its polyA tail, which—counter-intuitively—results in its stabilization. The mechanistic features of this unprecedented mRNA processing step resemble the initial Ire1-dependent nucleolytic step of RIDD but Bip1 mRNA then escapes further decay.

Our results shine new light on how ER homeostasis can be maintained and underscore the fascinating divergence of solutions that different species evolved to achieve this task.

The Physiological Role of RIDD in S. pombe

ER stress arises from insufficiencies in handling the protein-folding load in the ER lumen. Homeostasis, therefore, can be reestablished in two principal ways: increasing the capacity of the ER to handle the load—or decreasing the load to meet the capacity. Here we show that mRNA decay can serve as the sole means of resolving ER stress without transcriptional up-regulation of classical UPR target genes. The identified transcripts targeted for RIDD compose a subset of mRNAs, all encoding proteins that reside in or traverse the secretory pathway. Being translated by membrane-bound ribosomes, these mRNAs are therefore in an appropriate cellular location to meet activated Ire1.

As all proteins encoded by RIDD target mRNAs enter the ER lumen, their synthesis by definition contributes to the burden of the ER protein folding machinery. RIDD therefore helps reduce the protein-folding load. It is less clear however whether such reduction would have a major impact. Indeed, a back-of-the-envelope calculation
indicates that in *S. pombe* RIDD reduces the total protein influx into the ER by only 15%, even under the severe ER stress conditions explored here experimentally. This estimate derives from our ribosome footprinting data in normal versus ER-stressed cells: We scored the relative translational engagement of all mRNAs encoding proteins displaying signal sequences or transmembrane regions to estimate flux of newly synthesized polypeptides into the ER and calculated the impact of RIDD on this set (see Material and Methods). It is difficult to envision how a mere 15% reduction of bulk protein flux into the ER would suffice to alleviate an otherwise lethal ER stress.

It is possible that RIDD preferentially targets proteins that are particularly difficult to fold and hence might have a disproportional impact on the protein folding load in the ER lumen. Indeed, Ire1 may be localized to the vicinity of mRNAs encoding such proteins by interactions of its ER luminal unfolded protein sensing domain with portions of the nascent polypeptide chains that have entered the ER lumen, as previously proposed (14, 27). An alternative and not mutually exclusive view poses that RIDD qualitatively changes the gene expression profile. In support of this notion, we notice that the population of RIDD target mRNAs is highly selective. mRNAs encoding proteins involved in lipid metabolism are highly enriched (31% of RIDD target mRNA as compared to 6.7% in all ER-targeted mRNAs). Moreover, we find that RIDD targets encoding proteins involved in sterol metabolism are particularly enriched (13% as compared to 1.3%). How reduced sterol synthesis would counteract the toxic effects of ER stress remains unclear. One possible explanation would be that ER stress limits sterol exit through vesicular transport and a compensatory reduction in sterol synthesis becomes important to sustain basic ER functions, perhaps by maintaining appropriate membrane
fluidity (28, 29). In this way, RIDD (akin to other degradative pathways) could adjust basic metabolic parameters in the cell (30).

**RNA Cleavage and Recognition**

Previous work strongly suggests that Ire1 is the nuclease that initiates RIDD in metazoan cells (15, 16, 18). Our results provide two further lines of evidence in support of this view: first, we show that and Ire1 RNase active site mutant, *Ire1(H1018N)*, is unable to sustain cell growth on ER stress-inducing media. This mutation was designed to block catalysis while retaining hydrogen-bonding interactions of the amino acid side chain. Indeed, the equivalent single amino acid substitution in *S. cerevisiae* Ire1, Ire1(H1080N), reduces catalytic activity by >10⁵-fold. Otherwise, Ire1(H1080N) is indistinguishable from wild type Ire1, both in its oligomerization and structural properties as determined by crystallography (31). Second, we showed that cleaved RIDD target mRNAs carry a 2′,3′-cyclic phosphate group, which is a prerequisite for the ligation reaction (tRNA ligase) used in the genome-wide mapping of mRNA ends created upon ER stress. Ire1 and tRNA endonuclease (and the Ire1-family member RNase L found in mammalian cells) are the only cytoplasmic nucleases known to produce such products.

In *S. cerevisiae*, Ire1 has a single known substrate, *Hac1* mRNA (17). *Hac1* and *XBP1* mRNA have highly conserved and readily recognizable stem loop structures that demarcate the splice sites. Cleavage occurs at a universally conserved G always found at position 3 in the seven-base loop (20). This information is interchangeable between species: constructs derived from *S. cerevisiae Hac1* mRNA are properly spliced in mammalian cells, and yeast Ire1 recognizes and precisely cleaves *XBP1* mRNA-derived
substrates (32). We show that RIDD target mRNAs contain a short three-base UGC consensus at the Ire1 cleavage site where cleavage occurs after the G, consistent with cleavage specificity previously assigned to Ire1 (20).

Thus by contrast to our understanding of the RNA-elements directing Ire1-cleavage that initiates Hac1 and XBP1 mRNA splicing, the information that directs mRNAs into RIDD remains vastly underspecified. By chance, UGC triplets occur much more frequently in mRNAs than Ire1 cleavage sites. Therefore, the information that specifies an mRNA as an Ire1 substrate must require additional elements. Potential determinants may lie in sequence or secondary structure determinants that to date have escaped bioinformatics identification. We and others note that many of the identified cleavage sites lie in loops of potential hairpin structures (22, 33); however, the position of the scissile G in the loops is not conserved. These structures therefore do not provide a structurally plausible explanation. Alternatively, Ire1’s lumenal domain may become preferentially engaged with nascent polypeptide chains that display higher affinity and/or longer exposed peptide sequences, thereby selecting mRNAs co-translationally by recognizing features in the encoded protein. The concept of Ire1 recruitment, whether through interactions via the nascent chain or elements in the mRNA per se, is supported by our finding that mutation of one cleavage site (UG\C \rightarrow UC\U) in Gas2 mRNA gives rise to cleavage at alternative sites. These data indicate that local proximity rather than the RNA sequence surrounding the immediate cleavage site may guide substrate selection.

In order to stabilize the primary Ire1 cleavage products, we used mutant cells impaired in exosome function catalyzing 3’ \rightarrow 5’ RNA degradation (34, 35). Intriguingly,
we observed that Gas2 mRNA decay in Ski2Δ mutant strains was observed only transiently, peaking at 30 min after ER stress induction. This kinetic behavior suggests that clearance of Ire1-cleavage products by Ski2 may be a requirement for continued Ire1 activity. Thus, initiation of RIDD by Ire1 and further decay may be obligatorily coupled.

**Processing of Bip1 mRNA**

The observation that *S. pombe* Bip1 mRNA changes size upon ER stress dates back to 1992 (36). To date, Bip1 mRNA processing has not been observed in any other species. The phenotypic observation of the mRNA size shift has been deployed many times as an ER stress indicator (37), but, surprisingly, its origin has not been investigated.

As we suggest here, Bip1 mRNA is also cleaved by Ire1, yet escapes decay. The Ire1 cleavage site shares the same features described above for RIDD target mRNAs. During ER stress-induced processing, Bip1 mRNA loses a portion of its 3’ UTR and polyA tail. The resulting processed tBip1 mRNA is more stable and hence is present at an increased steady-state concentration. A plausible explanation for the increased stability of tBip1 mRNA is the loss of an RNA degron located in the severed portion of the 3’ UTR. tBip1 mRNA is actively translated with its ribosome density paralleling its increased abundance. Although polyA tails are generally linked to stability and translational efficiency, histone mRNAs, which likewise lack polyA tails, provide precedence for such an exception to the rule (38). Histone mRNAs terminate in a well-conserved 3’ stem-loop structure, which protects from exonucleolytic degradation. Proteins binding there functions akin to the polyA binding proteins found on other mRNAs to enhance histone mRNA translation by looping back to the 5’ cap structure (39).
Why \( tBip1 \) mRNA escapes decay remains to be explored. Possible explanations include the presence of secondary structure elements. Indeed, we find a predicted conserved hairpin structure at the 3’ termini of \( tBip1 \) RNAs in some fission yeasts (\( S. pombe, S. octosporus \) and \( S. cryophilus \)); however preliminary mutational analysis failed to validate its importance for mRNA stability in \( S. pombe \). An alternative possibility is that the 3’ end of \( tBip1 \) mRNA may be covalently modified. Such modification would need to be restricted to the 2’-OH, because the 3’-OH group is still accessible for modification by RNA ligase (Fig 3d). In this regard, \( tBip1 \) mRNA would resemble miRNAs, which are 2’-O-methylated, conferring resistance to degradation (40).

Our data show that \( Bip1 \) mRNA is the only mRNA in \( S. pombe \) in the expressed genome that is subject to this unique regulation. Why would such a singular mechanism have evolved exclusively for Bip1? From work in other species, Bip1 emerges as the most pleiotropically important and precisely controlled ER chaperone (41). Moreover, Bip1 holds a unique position in \( S. pombe \), where it is glycosylated (42). A recent comprehensive gene interaction map revealed that Ire1 in \( S. pombe \) clusters tightly with enzymes involved in the quality control cycle of glycosylated proteins, pointing toward a unique connection between glycosylation and ER stress (25). By contrast, corresponding E-maps in \( S. cerevisiae \) succinctly confirm the long-appreciated linear relationship between Ire1 and Hac1 (43). One may speculate that ER stress in \( S. pombe \), as in other species, enhances turnover of glycosylated proteins and that the regulation of \( Bip1 \) mRNA is beneficial to its stability by compensating for such loss (30).
Conclusions

From an evolutionary angle, the UPR in *S. pombe* provides an intriguing example of how molecular machines can be repurposed. While input (unfolded proteins, ER stress) and output (RNA cleavage) have been conserved, both detail and global consequences of downstream processes have been adapted to serve different needs. The UPR in both *S. cerevisiae* and *S. pombe* fulfills a cytoprotective role, yet the mechanisms of executing this task are opposed. In *S. cerevisiae*, folding capacity is increased via transcriptional up-regulation; in *S. pombe* the folding load is decreased and the ER is restructured. In metazoan cells, both modes of Ire1 activity are merged, and depending on condition and cell type can serve different purposes. RIDD can protect cells by removing major secretory protein loads, as it is the case in insulin secreting cells (23), or it can serve to activate apopototic pathways, as it is the case in cells experiencing prolonged and unmitigated ER stress (16).

It has always been puzzling how a strictly cognate system, such as the Ire1- and Hac1-mediated UPR regulatory pathway, would have evolved. While we cannot ascertain what represents the ancestral state, it is tempting to speculate that a broader mRNA degradation pathway preceded the development of the more specialized splicing mechanism. The prevalence of a much broader scope of Ire1 targets in *S. pombe* suggests that a primitive UPR may have served primarily as an ER-localized yet promiscuous RNA degradation system. Individual mRNA substrates would then have evolved appropriate affinities for the enzyme, rendering them more or less susceptible substrates. In this way, the stem/loop splice sites of *HAC1/XBP1* mRNAs could be the result of a long time optimization process: duplication of the cleavage site with concomitant
recruitment and repurposing of tRNA ligase would have culminated the UPR splicing reaction. In this view, *S. cerevisiae* emerges as the endpoint of an optimization process rather than an evolutionary precursor. By losing the more ancient RIDD function of the UPR, *S. cerevisiae* cell would have developed to rely exclusively on a more refined and more powerful transcriptional regulation program.
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Materials and Methods

Nomenclature

The unified convention used in this manuscript for genes and proteins is based on the treatment by Alberts, et al. in Molecular Biology of the Cell (5th edition, Garland Publishing), page xxxii. In brief, all genes are denoted in italics with a capitalized first letter. Mutant alleles are indicated by appending a descriptor to the gene name. Proteins are indicated in Roman letters.

Strains, plasmids and growth conditions

Standard media and genome engineering methods for fission yeast were used as described previously (44). Strains and plasmids used in this study are listed in Table S1. Briefly, reporter constructs were integrated into the Leu1 locus using plasmid pJK148. Mutant alleles were integrated by the pop-in/pop-out method using the integrative plasmid pJK210 (ura4) (45). All experiments were carried out in yeast extract complex media (YE5S) or in Edingburgh minimal media (EMM), supplemented with 0.225 mg/ml of L-histidine, L-leucine, L-lysine, adenine and uracil at 30°C, unless otherwise described. For pop-in/pop-out experiments, 5-FOA media containing 1 g/l 5-fluoro-orotic acid was used.

RNA analysis

Total RNA was purified by standard hot-phenol extraction (46). After precipitation RNA samples were re-suspended in DEPC-treated water and quantified by
spectrophotometry. Northern blotting, electrophoresis, labeling, analysis and quantification were performed as described (47).

**Protein analysis**

Cells were cultured in YE5S media. Between 5 and 10 OD units were collected by centrifugation and snap-frozen. Cell pellets were thawed on ice, re-suspended in 200 µl of lysis buffer (8 M urea, 50 mM HEPES pH 7.4) and lysed in a glass bead mill (5 min at 4°C). After adding 20 µl of a 25% SDS solution, samples were incubated at 65°C for 5 min. The lysates were collected by piercing the bottom of the tubes with a syringe needle and clarified by re-centrifugation (1,000 rpm for 10 sec). Total protein concentration was determined by a standard bichromic acid (BCA) assay (manual instruction, Pierce Biotechnology). 10 µg of lysate per lane were electrophoresed on SDS-polyacrylamide gradient gels (4%-15%, BioRad). The separated proteins were subsequently transferred to PDVF membranes at 200 mA for 1 h. Blots were blocked with 5% milk in Tris-buffered saline (10 mM Tris, 150 mM NaCl, 0.1% Tween-20) and incubated with primary antibodies overnight at 4°C. Antibodies and dilutions: rabbit polyclonal anti-Kar2 (1:5000), mouse monoclonal anti-RNA polymerase II carboxy-terminal domain (CTD) repeat (Abcam ab817) (1:8000 dilution). Blots were washed and incubated with Li-Cor fluorescently-coupled secondary antibodies (1:5,000) for 30 min. Immunoreactive bands were identified using a Li-Cor infrared scanner, and processed with the Odyssey software package.
**Bip1 mRNA linker-ligation and 3’ rapid amplification of cDNA ends (RACE)**

Total RNA (10 µg) from treated or untreated (2 mM DTT, 1 h) WT cells were incubated with 25 units of polynucleotide kinase (PNK) to remove 2’,3’-cyclic phosphates for 1 h at 37°C. After deactivating the enzyme (75°C for 10 min), dephosphorylated RNA was precipitated and re-suspended in 6 µl DEPC-treated water. After denaturing the RNA at 80°C for 5 min, a linker-ligation reaction was performed in the presence of dephosphorylated, denatured RNA, T4RNA Ligase II (NEB), RNase inhibitor (40 Units), DMSO, 50% PEG and 1 µg of 5’ pre-adenylated linker (5'AppCTGTAGGCACCATCAAT/3ddC3', as described (48). Reverse transcription was performed using a reverse complement DNA-oligonucleotide to the linker sequence. The 3’ RACE reaction was performed as described (49). Nested Bip1-PCR products were purified from ethidium-agarose gels and sequenced.

**qPCR analysis of Gas2 mRNA reporter**

Total RNA (1 µg) was reverse-transcribed with random hexamers. 1% of the resulting cDNAs was employed for real-time PCR reactions utilizing SYBR green. The reactions were run and analyzed in a DNA Engine OPTICON 2 using the BioRad Opticon Monitor 3.0 software.

**Growth assay and colony forming unit assay for Bip1 3’UTR mutant**

WT and Bip1(ΔTTAACTGGTGC) mutant cells were grown overnight in (YE5S) media. The next morning cultures were diluted and grown until they reached an OD of 0.25. ER-stress was induced with tunicamycin as indicated. After 3 h, stressed cells were
washed four times with pre-warmed YES5 media to remove the drug, the culture was readjusted to an OD of 0.25, and incubated in a 24 well plate (1 ml per well) over 32 h at 32°C. The OD was measured every 10 min in a microplate reader (Synergy 4 BioTeK). The colony forming unit assay was performed by plating washed cells at different dilutions on solid media (YE5S) and incubated for 3 days at 30°C. Colonies were counted from the dilutions series. Untreated cells served as a control.

*Bip1* mRNA half-life measurement

To determine the half-life of *Bip1* mRNA, we constructed an integrative plasmid (pJK148) containing the open reading frame and 3’ UTR of *Bip1* mRNA under the control of a thiamine-regulated *Nmt1* promoter. The construct included the *Nmt1* 5’ UTR. Cells were grown in EMM complete media (without any thiamine) for 24 h. Cells were then diluted into fresh EMM complete media and re-grown to an OD of 0.3. After inducing ER stress with tunicamycin (0.25 µg/ml) for 1 h, thiamine was added to 15 µM to block transcription of the *Nmt1* promoter (50). Samples were processed at indicated time points and subjected to Northern blot analysis. A DNA probe complementary to the 5’UTR of nmt1 was used for detection.
RNA-deep-sequencing sets:

Figure 1 – source data:

- oligo-dT-enriched mRNA setI (including: WT, WT +DTT (2 mM, 1h) and $Ire1\Delta$+DTT (2 mM, 1h))

- oligo-dT-enriched mRNA setII (including: WT, WT +DTT (2 mM, 1h) and (2 mM, 1h))

- total (-depleted rRNA) RNA set I (including: WT, WT +DTT (2 mM, 1h)

- total (-depleted rRNA) RNA set II (including: WT, WT +DTT (2 mM, 1h) and $Ire1\Delta$+DTT (2 mM, 1h))

Figure 2 – source data

- 2’,3’-cyclic phosphate 3’end mapping setI ($Ski2\Delta$ (2 mM DTT, 30min) $Ire1\Delta$ $Ski2\Delta$ (2 mM DTT, 30min))

RNA isolation for deep-sequencing library

As indicated, one of three methods was utilized to isolate RNA with specific chemical properties: polyA+ tail enrichment, rRNA depletion, or 3’ end 2’,3’-cyclic phosphate enrichment. Total RNA was prepared from cells by the hot acid phenol method (46), and subsequent enrichment performed. PolyA+ mRNA was purified by two sequential rounds of enrichment using oligo-dT DynaBeads (Invitrogen) according to the manufacturer’s instructions. rRNA depletion was performed by first depleting all abundant RNAs smaller than 200nt using the modified protocol for isolating only large RNAs provided in the mirVana miRNA Purification Kit (Ambion) followed by two
rounds of subtractive hybridization using the Ribominus Eukaryote Kit for RNA-Seq (Invitrogen), according to the manufacturer’s instructions. To sequence 2’,3’-cyclic-phosphate cleavage products purified tRNA ligase (a kind gift from J.R. Hesselberth) was used to selectively ligate an RNA linker to all 2’,3’-cyclic phosphates in total RNA as previously described (26). PolyA⁺-enriched and rRNA-depleted samples were randomly fragmented under basic conditions, precipitated by standard methods, and ~50 nt fragments were size-selected by polyacrylamide gel electrophoresis as described in (51).

**Ribosome footprint isolation for polysome profile and deep-sequencing library**

Ribosome footprints were isolated as described in (51), with minor modifications. Briefly, 750 ml mid-log yeast cultures (+/- 30 min 2 mM DTT treatment) were harvested by filtration without the addition of cycloheximide, and were immediately flash frozen. Frozen cells were cryogenically lysed in the presence of 3 ml of frozen polysome lysis buffer (20 mM Tris pH 8.0, 140 mM KCl, 1.5 mM MgCl₂, 100 µg/ml cycloheximide, 1% Triton) on a Retsch MM301 mixer mill, and thawed lysates were subsequently clarified by centrifugation as described. ~50 A₂₆₀ units of clarified lysate was treated with 750 U of *E. coli* RNase I (Ambion) for 1 hour on ice to minimize 80S degradation. Monosomes were collected from sucrose density gradients (10-50% w/v, prepared in polysome lysis buffer: 20 mM Tris pH 8.0, 140 mM KCl, 5 mM MgCl₂, 100 µg / ml, cycloheximide, 0.5 mM DTT, 20 U / ml SUPERase·In) as described, and undigested control samples were loaded to generate polysome profiles shown in Figure 4B. RNA from monosome or polysome fractions was isolated using the hot acid phenol method. For ribosome profiling, 28-34 nt RNA fragments from the monosome fraction were size-selected by gel electrophoresis as described above.
**Library preparation**

Deep sequencing libraries were constructed as described in (48). Briefly, size-selected mRNA (polyA\(^+\) and -rRNA) or ribosome footprints were 3’-dephosphorylated with T4 polynucleotide kinase (NEB). 3’-dephosphorylated RNA was ligated to a preadenylated miRNA cloning linker (IDT, Linker #1) using T4 RNI2(tr) (NEB) rather than enzymatically polyadenylated. Subtractive hybridization of rRNA contaminants was not performed. Ligated samples were directly reverse transcribed using SuperScriptIII (Invitrogen), circularized using CircLigase (Epicenter), and PCR amplified.

**General RNA-Sequencing data processing**

Data from deep-sequencing analyses of total (-ribosomal RNA) RNA, polyA\(^+\) enriched mRNA and ribosome foot-printing were collected and the resulting sequences were aligned using the following method: the linker sequences at the 3’ ends were removed prior to alignment using SOAP2.20, allowing a maximum of 2 total mismatches (52). Ribosome footprint reads were assigned to a specific A-site nucleotide by an offset of +15 from 5’ end of the read (only for ribosome foot-printing data set). All reads aligned to rRNA and tRNA were removed. To align intron-exon junction reads, all reads with no alignment against *S. pombe* genomics sequences were re-aligned against a sequence library of *S. pombe* processed protein-coding transcripts. All the alignments were performed against the most recent version of the *S. pombe* genome (the [www.genedb.org/genedb/pombe/](http://www.genedb.org/genedb/pombe/)). The raw sequencing data will be available for download at NCBI GEO.
Quantification of mRNA abundance

Biological replicates (set I and set II, where available) were combined to increase read coverage. After combining sets, mRNA (ORF) transcripts with fewer than 100 reads before normalization were excluded. Next, the passed mRNAs (ORF) were normalized to reads per million total reads (rpM). Because the total number of reads may not reflect the total RNA production correctly (53), the observed count for gene \( g \) in condition \( k \) need to be normalized to the total RNA production to calculate the fold-change between two conditions. We used the following method to estimate the correct RNA abundance for given RPM values. Given conditions \( k \) and \( r \), we calculated the expected RNA abundance of condition \( k \) given the RPM value in condition \( r \) with the following equation:

\[
x_{k,r,g} = 2^{(a_{k,r} \log_2(x_{r,g}) + b_{k,r})}
\]

\( x_{r,g} \) is the RPM value for gene \( g \) in the reference condition, \( x_{k,r,g} \) is expected RPM estimated with linear regression between the RPM values from condition \( k \) and \( r \). \( a_{k,r} \) and \( b_{k,r} \) are the coefficients of the linear regression where

\[
a_{k,r} = \frac{\sum(Y_{k,g} - \overline{Y}_k)(Y_{r,g} - \overline{Y}_r)}{\sum(Y_{r,g} - \overline{Y}_r)^2}
\]

\[
b_{k,r} = \overline{Y}_k - a_{k,r} \overline{Y}_r
\]

in which \( Y_{k,g} = \log_2(x_{k,g}) \), \( Y_{r,g} = \log_2(x_{r,g}) \).

For the above linear regression, genes with \( M_g = \frac{x_{k,g}}{x_{r,g}} \geq 10 \) or \( \leq 0.1 \) were removed from the data set to estimated \( a_{k,r} \) and \( b_{k,r} \). The fold change between condition \( k \) and \( r \) is then calculated as: \( F_{g,k,r} = \frac{x_{k,g}}{x_{k,r,g}} \), which is the RPM value for gene \( g \) under condition \( k \) divided by the expected RPM estimated above.
2',3’-cyclic phosphate 3’ end RNA mapping and Ire1 cleavage site motif determination

Sequence read alignments from the 2’,3’-cyclic phosphate mapping data were performed as for the RNA-Seq reads described above. The design of the library (3’ end mapping) made it necessary to align the reads to the opposed strand of the gene. To map putative Ire1-dependent cleavage sites with high stringency, we identified the positions within each transcript containing more than 4 reads in RNA derived from the Ski2Δ sample and zero reads in RNA derived from the Ire1Δ Ski2Δ sample. We identified by this method 4027 putative Ire1-dependent cleavage sites in the genome. By using a less stringent criterion (by taken the ration between Ski2Δ sample and Ire1Δ Ski2Δ sample: we allowed a ratio of 5 or more). By this criterion we identified 4134 putative Ire1-dependent cleavage sites. The overlap between the two methods is 97%. We continued our analysis with the more stringent criteria of only allowing zero reads in Ire1Δ Ski2Δ sample. To identify an overrepresented consensus sequence, we first extended the sequences 9 nt upstream and downstream of the potential cleavage sites. Furthermore, we used a position weight matrix (PWM), which was generated from these sequences by weighting each sequence with the reads from the Ski2Δ sample. By using all annotated genes and the corresponding putative cleavage sites, we could not identify overrepresented sequences. The same held true for the ER-target mRNAs set (N=1014). By contrast, a strong consensus motif (Figure 2e) emerged when we mapped the putative cleavage sites in our set of 39 Ire1- and ER stress-dependently two-fold down-regulated transcripts.
Estimation of protein flux into the ER

We scored ribosome footprint reads per kilobase (to normalize for length of open reading frames) per million reads (to compare different conditions) for the set of mRNAs, encoding proteins predicted to enter the ER as described above (see Table 1).
Figure 1. The UPR in fission selectively down-regulates ER-targeted mRNA

(a) Phylogenetic tree showing the components of the UPR in yeasts. The presence of recognizable orthologs of Ire1 and Hac1 is indicated. (b) Viability assay by serial dilution of wild type, \textit{Ire1}\textit{Δ} and \textit{Ire1(H1018N)} cells spotted on solid media with or without 0.03 µg/ml of the ER stress inducer tunicamycin (Tm). Plates were photographed after 3 day of growth at 30°C. (c) Strand-specific polyA\(^+\) enriched mRNA-Seq analysis of annotated ORFs. The plot indicates the fold change (log\(_2\)) of transcript abundance in DTT-stressed \textit{Ire1Δ} cells (2 mM DTT, 1 h) compared to DTT-stressed wild type cells (2 mM DTT, 1 h) in the x-axis, and transcript abundance in unstressed wild type cells compared to DTT-stressed wild type cells (2 mM DTT, 1h) in the y-axis. Symbol sizes indicate abundance classes for each mRNA (reads per kilobase). Transcripts encoding proteins with a signal sequence or transmembrane segment are colored red, all other transcripts are colored blue (Fig 1 - source data). (d) DTT-dependent and Ire1-dependent expression changes of transcripts displaying a signal sequence or a transmembrane domain. The skew of the left tail of the distribution indicates an enrichment \((P < 1 \times 10^{-20})\) of down-regulated mRNAs. Coloring is as in Figure 1d. (e) Distribution of gene-ontology (GO) annotations for Ire1-dependent down-regulated mRNAs. Percentages indicate genes within a particular GO category in relation to the total number of genes that have a GO annotation \((N = 39)\)(see Figure 1 – S3 for annotated list of genes).
Figure 1.
Figure 2. Ire1 cleaves down-regulated mRNAs at specific sequences

(a) Northern blot of total RNA extracted from wild type, Ire1Δ, Ski2Δ and double mutant ER stressed Ire1Δ Ski2Δ cells (2 mM DTT, 1 h). A probe complementary to the 5’ UTR of Gas2 was used to detect cleavage products. The triangle and asterisk indicate two different mRNA cleavage products. (b) Northern blot of total RNA extracted from ER-stressed Ski2Δ cells (2 mM DTT). (c) Viability assay by serial dilution of wild type, Ire1Δ, Ski2Δ and Ire1Δ Ski2Δ cells spotted on solid media with or without ER stress as in Figure 1b. (d) RNA-sequence read density map of the Gas2 locus derived from 3’ end deep-sequencing data. Library was generated by ligating a DNA-linker using tRNA ligase to 3’ end mRNAs with 2’,3’-cyclic phosphates in Ski2Δ and Ire1Δ Ski2Δ ER-stressed cells (2 mM DTT, 30 min). The arrows indicate two Ire1-dependent cleavage sites. The U6 snRNA locus was used as a positive control (Figure 2 – source data). (e) Ire1 RNA sequence recognition motifs generated by deep-sequencing analysis of tRNA ligase-generated RNA libraries of 39 mRNA targets down-regulated two fold or more in an Ire1-dependent manner. The resulting position weight matrices are illustrated as a logo. The dotted line indicates the cleavage site. (f) Real-time qPCR of a chromosomally integrated reporter containing the coding sequence of Gas2 under the control of the Nda2 (tubulin) promoter and including the UTRs of Nda2. A time course after DTT addition (2 mM) is shown. Endogenous Nda2 was used as a normalization control. Error bars: standard deviation. (g) Northern blot analysis of total RNA extracted from Ski2Δ and Ire1Δ Ski2Δ cells carrying a mutant version of the reporter indicated in (f) were the putative Ire1 cleavage site (▲, UG\CU-> UC\UU) was mutated. Note that the band labeled × migrates distinctly faster, as shown by scan on the right.
Figure 2.
Figure 3. Ire1 truncates Bip1 mRNA within the 3’ UTR

(a) Northern blot analysis of total RNA extracted from wild type and Ire1Δ cells untreated or treated with tunicamycin (1 µg/ml), and hybridized with a probe complementary to the ORF of Bip1 mRNA. Right panel: quantitation normalized to Pgtk mRNA. (b) The abundance of a GFP mRNA driven by the Bip1 promoter (black) compared to endogenous Bip1 mRNA was determined as a time course after DTT (2 mM) addition by quantitative Northern blotting. (c) Wild type cells bearing a construct encoding the Nmt1 5’ UTR, Bip1 ORF and Bip1 3’ UTR driven by the Nmt1 promoter were pre-treated with tunicamycin (0.25 µg/ml, 1 h). At different time points after thiamine (15 µM) addition (to effect transcriptional shut-off of the Nmt1 promoter), RNA was extracted and analyzed by Northern hybridization. Blots were probed for the Nmt1 5’ UTR. Nmt1-Bip1 mRNA and Nmt-tBip1 mRNA were quantitated and normalized to the unspecific band (asterisk). (d) RNA-sequence read density map of the Bip1 locus derived from mRNA-enriched (ribosome depleted) RNA in wild type cells untreated or treated with DTT (2 mM DTT, 1 h; left panels). Data are representative of one of two biological replicates. Single nucleotide resolution of the 3’ terminus of Bip1 mRNA determined by 3’ RACE (right panels). (e) Mutational analysis of the Bip1 mRNA cleavage site by Northern blotting. Total RNA was extracted from wild type, Ire1Δ or cells carrying mutations in the Bip1 3’ UTR mRNA. Cells were treated with 2 mM DTT, 1 h or left untreated as indicated. The fold-changes indicate Bip1 mRNA abundance relative to that of Pgtk mRNA. (f) Strand-specific, mRNA enriched (after removal of ribosomal RNA) deep-sequence analysis of annotated ORFs (y-axis) compared to strand-specific polyA+ enriched mRNA deep-sequence analysis of annotated ORFs (x-axis) (see Figure 1-
source data). The plot indicates the ratio of transcript abundance in unstressed versus
DTT-stressed (2 mM DTT, 1 h) wild type cells. Symbol sizes and colors are as described
in Figure 1c.
Figure 4. *tBip1* mRNA is translated and is important for fitness during ER stress

(a) Northern blot analysis of the distribution of total or *tBip1* mRNA in polyribosomes from extracts of unstressed or ER-stressed (2 mM DTT, 1h) cells. Fractions 11, 12, 13 of the sucrose gradients (lower panels) were analyzed by Northern blotting (upper panels).

(b) Ribosome footprints (as described in Material and Methods) of *Bip1* mRNA in unstressed or ER stressed cells. The region that depicts ribosome density preceding the *Bip1* ORF is shown enlarged in Figure 4 - Figure Supplement 1 (Figure 4 – source data 1).

(c) Cell growth of wild type cells and cells carrying a deletion of *Bip1* mRNA cleavage sites (*Bip1*(ΔTTAACTGGTGC)). Cells were treated with tunicamycin (0.5 µg/ml) for 3 h and then recovered from ER stress by washing out the drug and re-seeding in warm fresh media. Optical density (OD) at 660 nm was measured immediately afterwards in 10 min intervals.

(d) Viability assay of the same cells as in (c). The percentage of viable cells was determined by counting the number of colony-forming units (CFU) after growth for 3 h at varying tunicamycin concentration.
Table 1. Ribosome footprints of mRNAs encoding proteins predicted to enter the ER.

<table>
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<tr>
<th>all ER-targeted mRNAs ribosome occupancies (N=1014 mRNAs)</th>
<th>rpkms</th>
<th>ration normalized to WT-DTT</th>
<th>[%]</th>
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<td>100</td>
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<tr>
<td>WT (+DTT)</td>
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<td>ΔIRE1 (+DTT)</td>
<td>90166</td>
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<td>107.9315298</td>
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</table>

rpkm = reads per kilo-base of transcript per million reads
**Figure 1 – S1.** Alignment of DNA binding domain of Hac1 (bZIP) homologues in different yeast species.

**DNA binding domain of Hac1 homologs**

<table>
<thead>
<tr>
<th>Species</th>
<th>Alignment of DNA binding domain</th>
</tr>
</thead>
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Figure 1–S2. Plot depicting ER-targeted mRNAs abundance $[\log_{10}]$ (reads per million) versus DTT-dependent expression changes $[\log_2]$ for wild type cells.
Figure 1 – S3. Ontology of genes which are down-regulated more than 2 fold *Ire1- and DTT-dependent.

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<th>gene</th>
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<th>product</th>
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ss = signal sequence
TM = transmembrane domain

N=38

lipid metabolism
sterol metabolism (part of lipid metabolism)
ER function
Trafficking
Plasma membrane
other functions
poorly characterized

61
Figure 2 – S1. Northern blot of total RNA isolated from wild type, \textit{Ire1}Δ, \textit{Ski2}Δ and double mutant \textit{Ire1}Δ \textit{Ski2}Δ ER stressed cells (2 mM DTT, 1 h). A probe complementary to the 5’ UTR of \textit{Yop1} was used to detect cleavage products. The arrows indicate Ire1-dependent mRNA cleavage products.
**Figure 2 – S2.** Comparison of ER stress-dependent down-regulation of endogenous *Yop1* mRNA (2 mM DTT, 1 h: deep-sequencing) and reporter *Gas2* mRNA (from qPCR: 2 mM DTT, 1h; see also Figure 2f).
**Figure 3 – S1:** Northern blot analysis of wild type cells bearing a construct expressing a fusion protein of GFP preceded by the Bip1 signal sequence. The constructs includes the Bip1 3’ UTR. Expression was driven by the Nmt1 promoter. Cells were untreated or treated with DTT (2 mM DTT, 1h) as indicated. To abolish targeting of the mRNA to the ER, three hydrophobic leucine residues in the signal sequence were replaced by three charged arginine residues. MKKFQLFSILSYFVALLLPMAFA (WT) to MKKFQRFSLSYFVARLLPMAFA. Silent mutations were created by changing one nucleotide in each three leucine residues (above) without changing the amino acid.
**Figure 3 – S2:** Sequencing read coverage of the 3’ end nucleotide positions in *tBip1* mRNA from derived from mRNA enriched by subtractive hybridization against rRNA (Ribominus™ kit, Invitrogen kit). Cells were treated with DTT (2 mM, 1 h).
Figure 4 – S1: Zoomed-in ribosome occupancy profile of Bip1 mRNA around the start AUG codon of wild type cells. Putative non-canonical uORFs are highlighted in both Bip1 and tBip1 mRNA derived from untreated and DTT-treated (2 mM, 1 h) cells.
**Figure 4 – S2.** Upper panel: Western blot of Bip1 and, as a loading control, RNA polymerase II CTD repeat (RNAPII). Wild type and *Ire1Δ* cells were treated with tunicamycin 0.5 µg/ml and samples were taken at indicated time points. Lower panel: Quantification of Western blotting with values normalized to RNAPII.

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**Legend:**
- *wild type*
- *ire1Δ*
**Figure 4 – S3.** Viability assay by serial dilution of wild type, *Ire1Δ* and different *Bip1* mRNA cleavage mutants spotted on solid media with or without 0.03 μg/ml of the ER stress inducer tunicamycin. Plates were photographed after 3 day of growth at 30°C.
References


35. Anderson JS, Parker RP. The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex. The EMBO journal. 1998;17(5):1497-506. Epub 1998/04/18.


Chapter 3

Characterization, Purification and *in vitro* Analysis of Fission Yeast Ire1
Introduction

Chapter 2 outlines the very beginnings of our characterization of the Unfolded Protein Response (UPR) in fission yeast. We have found that while Ire1 is highly conserved from *S. cerevisiae* to *S. pombe*, fission yeast utilizes Ire1 in a very different way to restore endoplasmic reticulum (ER) homeostasis. Ire1 in fission yeast is responsible for activating two separate posttranscriptional RNA regulatory events designed to downregulate protein influx into the ER, rather than utilizing the transcriptional upregulation of ER components seen in budding yeast. At the core of this mechanism is the enzymatic function of Ire1.

Ire1 is a conserved single pass transmembrane protein that lies within the membrane of the ER. While Ire1 is found in all eukaryotes, the mechanism of Ire1 function has been most extensively studied in the budding yeast *S. cerevisiae*. In budding yeast, it is a 127 kDa protein consisting of three domains; a stress sensing domain resides in the lumen of the ER, while a serine/threonine kinase domain and an endoribonuclease domain are on the cytoplasmic side of the ER membrane. When the UPR is induced by ER stress, Ire1 is responsible for signaling across the membrane of the ER to the nucleus where a vast transcriptional response is activated to expand ER protein folding capacity (Cox et al., 1993; Travers et al., 2000). Ire1 senses protein-folding stress within the lumen of the ER by directly binding to accumulated unfolded proteins (Credle et al., 2005; Gardner and Walter, 2011). It relays this signal to the nucleus through the unique cytoplasmic splicing of the *HAC1* mRNA (Cox and Walter, 1996; Rüegsegger et al., 2001; Sidrauski et al., 1996). Once activated, the endoribonuclease domain of Ire1 cleaves conserved hairpin loops within the unspliced cytoplasmic *HAC1* mRNA, which
removes an intron that serves to inhibit translation (Gonzalez et al., 1999; Rüegsegger et al., 2001). The tRNA ligase, Trl1, then joins the liberated exons to form the translationally competent spliced \textit{HAC1} mRNA. The Hac1 transcription factor is then produced and travels to the nucleus to activate genes encoding for chaperones, protein folding enzymes and lipid biosynthetic machinery in an effort to restore ER homeostasis (Chapter 1, Figure 1).

As Ire1 is the most conserved branch of the UPR, it was no surprise that Ire1 also mediates the UPR in fission yeast. In fission yeast, we have shown that Ire1 is required for cell survival during conditions of UPR stress and, more specifically, this survival also required the RNase domain of the enzyme (Chapter 2). However, unlike its counterpart in budding yeast, Ire1 does not initiate a signaling mechanism to induce a transcriptional response. Instead, Ire1 is required to downregulate a specific population of mRNAs in response to UPR stress (Chapter 2) in a process reminiscent of regulated Ire1-dependent decay (RIDD) observed in metazoan cells (Hollien et al., 2009; Hollien and Weissman, 2006). Additionally, we have shown that Ire1 is required for the truncation and subsequent stabilization of the \textit{Bip1} mRNA (Chapter 2). Despite these significant observations, many areas of investigation remain. We have not yet shown that Ire1 is the protein responsible for direct cleavage of \textit{Bip1} or the downregulated mRNAs. It is also unclear how Ire1 selects which mRNAs to target or why the \textit{Bip1} mRNA experiences such a different fate than other Ire1 targets in fission yeast. Additionally, fission yeast and budding yeast Ire1 proteins appear to be recognizing a slightly different consensus sequence and cleavage occurs at a different base. We do not yet understand the structural reasons for such a difference in RNase activity.
Here I will outline preliminary efforts to further characterize Ire1. We have begun to study the interchangeability of *S. pombe* Ire1 with *S. cerevisiae* Ire1. Ire1 in fission yeast has been tagged and attempts have been made to purify the cytosolic domain of *S. pombe* Ire1. Further characterization and purification of *S. pombe* Ire1 will open up a new world of biochemical experiments that can be done to further explore the mechanism of UPR induction in fission yeast.

**Materials and Methods**

*Plasmid and Yeast Strain Construction*

Plasmids used in this study are listed in Table 1. Yeast strains used are listed in Table 2. All oligonucleotide primer sequences can be found in Table 3. To clone *S. pombe* Ire1 the gene was PCR amplified from purified *S. pombe* genomic DNA using *Pfu* TURBO (Strategene) using oligos oMD291 and oMD292. These oligos insert *KpnI* and *SacI* sites flanking the gene for easy cloning. Gel purified the 5,749 bp *Ire1* PCR product from 0.7% agarose gel in 1xTAE. Cloned *Ire1* PCR product into pJK210 vector using *KpnI* and *SacI* sites to make plasmid pMD50 (pJK210-*Ire1*). Sequenced to confirm *Ire1* sequence.

To utilize the superior homologous recombination abilities of *S. cerevisiae*, wildtype *S. pombe* Ire1 was subcloned from the *S. pombe* vector pJK210 into the *S. cerevisiae* expression vector pRS316. This allows for easy gap repair in *S. cerevisiae*. Accomplished this by cutting pMD50 with *SacI, KpnI and Stul*. *Stul* cuts the pJK210 vector backbone in half. Without this step, the vector backbone is the same size as the desired *Ire1* insert. Gel purified *Ire1* insert from 1% agarose gel in 1xTAE. Cloned
purified *Ire1* insert into pRS316 using *KpnI* and *SacI* restriction sites to create pMD54 (pRS316-*Ire1*).

Intron was removed from *S. pombe Ire1* in pMD54 using a single oligo mutagenesis technique (Makarova et al., 2000). Used primer oMD344 to create plasmid pMD84.

Used gap repair in *S. cerevisiae* to place a 3x flag tag in 3 different locations in *S. pombe Ire1* as follows: 1. the C terminus 2. the linker region 3. the T loop region. Made 3 different 3x flag tagged constructs to be integrated into the various desired regions. Used the following oligos each containing the 3X flag tag and flanking regions homologous for the area in *Ire1* for proper integration: for the C terminus- oMD329 and oMD330, for the linker region- oMD331 and oMD332, and for the T loop region- oMD333 and oMD334. Mixed equal amounts of each oligo into 3 separate reactions. Annealed and Extended using ExTaq (Takara) by denaturing 94°C for 30 seconds, annealing 49°C for 1 minute and extending 72°C for 20 seconds. Repeated 5 cycles. Linearized and gel purified pMD54 in 3 different locations for each individual tag placement as follows: C terminus- *XbaI*, linker- *SgrAI*, and T loop- *HpaI*. Transformed appropriate 3xflag tag with the corresponding linearized pMD54 plasmid into WT *S. cerevisiae* W303-1B strain (ATCC). Purified plasmids from *S. cerevisiae* and transformed into *E. coli*. Purified and sequenced plasmids from *E. coli* to check sequence and insert. This created pMD76 (pRS316-*Ire1*-3xflag-C terminus), pMD77 (pRS316-*Ire1*-3xflag-T loop), and pMD78 (pRS316-*Ire1*-3xflag-linker). Subcloned the *Ire1*-3xflag tagged inserts from the pRS316 vector backbone into the pJK210 *S. pombe* vector using the *KpnI* and *SacI* restriction
sites. This created pMD79 (pJK210-Ire1-3xflag-C terminus), pMD80 (pJK210-Ire1-3xflag-T loop), and pMD81 (p JK210-Ire1-3xflag-linker).

Linearized pMD79, pMD80 and pMD81 within the Ire1 gene insert and integrated into the Ire1 locus of WT S. pombe strain yMD76. Selected integerants on SD-ura plates. Used a loop-in-loop out technique by growing cells in YE5S media overnight and plating on 5-FOA plates to loop out Ura4 gene. Screened colonies by colony PCR and restriction digest to confirm the presence of the 3xflag tagged Ire1 at the Ire1 locus. This yielded yMD82 (Ire1-3xflag-C terminus), yMD83 (Ire1-3xflag-T loop), and pMD84 (Ire1-3xflag-linker).

Flow Cytometry

Plasmid containing S. pombe Ire1 without its intron (pMD86) was transformed into S. cerevisiae cells harboring an integrated HAC1 splicing reporter (Pincus, 2010). Cells were grown in selective media to maintain the plasmid. The UPR was induced and flow cytometry to measure splicing were performed as described (Pincus, 2010).

Western Blotting

Grew overnight liquid cultures in YE5S media at 30°C. Harvested 1.5 OD$_{600}$ units of each of the cells and froze pellets in liquid nitrogen. TCA precipitated protein as follows. Resuspended pellet in 150µl of Solution 1 (1.85M NaOH, 7.4% BME) and incubated on ice for 10 minutes. Added 150µl 50%TCA and incubated on ice for 10 minutes. Centrifuged for 2 minutes at maximum speed at 4°C. Removed supernatant and washed pellet in 1ml acetone. Resuspended pellets in 100µl of 1x protein loading buffer.
Boiled protein for 5 minutes and loaded 10µl on 10%, 10 well mini PROTEAN TGX gel (BioRad). Ran gel in 1x Laemmli buffer at 200V for 30 minutes and transferred protein to 0.45µM nitrocellulose membrane. Blocked membrane in 5% milk/PBST overnight at 4°C. Incubated membrane in anti-Flag tag antibody (Sigma, M2, F3165) diluted 1:2000 in 5% milk/PBST for 1 hour at room temperature. Washed in PBST. Incubated in anti-mouse secondary antibody (Pierce) diluted 1:50,000 in 5% milk/PBST for 1 hour at room temperature. Developed with Dura ECL (Pierce) and exposed to film. Alternatively, SF21 insect cells were lysed directly into loading buffer and detected as described above with the anti-HIS antibody (AbCam, 3H2201)

**Plate Growth Assays**

To assay growth, *S. pombe* strains were grown overnight in liquid cultures of YE5S rich media at 30°C. They were diluted to an OD<sub>600</sub> of 0.05 and allowed to grow for 6 hours at 30°C. Liquid cultures were diluted to an OD<sub>600</sub> of 0.15 in a sterile 96 well plate and diluted 1:5 in 6 sequential columns. Used frogging tool to spot onto YE5S, 0.025µg/ml tunicamycin, 0.05µg/ml tunicamycin and 0.1µg/ml tunicamycin containing plates. Grew at 30°C.

**Immunoprecipitation of Ire1-3xFLAG**

The fission yeast strain yMD82 containing Ire1 tagged at its C-terminus with 3xFLAG integrated into the Ire1 genomic locus was grown in YE5S media. A control *S. cerevisiae* strain containing Ire1 tagged at its C-terminus with 3xFLAG 6xHis integrated into the genomic locus of BY4741 (Gardner and Walter, 2011) was grown in YPD. Cells
were grown to an OD_{600} of 0.5 and the UPR was induced with the addition of 2mM DTT for 1 hour. Induced cells were harvested by filtration and directly frozen in liquid nitrogen. IP buffer (20mM HEPES pH 7.4, 250mM NaCl, 2mM Mg(OAc)\textsubscript{2}, 2% Triton X-100, COMPLETE protease inhibitor (Roche)) was frozen dropwise into liquid nitrogen. Frozen cells were combined with frozen IP buffer and lysed in a Retsch Mixer Mill for a total of 5 3 minute intervals at 15Hz. Cell debris was cleared from lysate by centrifugation at 4000xg for 15 minutes. Cleared supernatants were incubated with Invitrogen Dynabeads M270 conjugated to α-FLAG M2 antibodies (Sigma). The immunoprecipitation was incubated at 4˚C for 4 hours and then beads were washed with IP buffer including 0.05% Tween-20. Ire1-3xFLAG protein on beads was then used directly for \textit{in vitro} cleavage assays of either \textit{in vitro} transcribed P\textsuperscript{32} body labeled 3’UTR of Bip1 mRNA or HAC1\textsuperscript{U} 508 RNA as previously described (Gonzalez and Walter, 2001).

\textbf{Cloning of Ire1 into Protein Expression Vectors}

PCR amplified the cytoplasmic domain of \textit{S. pombe Ire1} from pMD84 as the PCR template using oMD446 and oMD447. This amplified a 1,371 bp fragment coding sequence of Ire1 lacking the intron. oMD446 and oMD447 amplify nucleotides 1849-3220. These oligos insert \textit{BamHI} and \textit{NotI} restriction sites flanking the coding region. Cloned the PCR product into pGEX-6P-2 to create pMD95. The resulting construct codes for an N-terminally GST tagged Ire1 cytoplasmic domain from amino acid 617-1072 of the \textit{S. pombe} protein and included its own stop codon.
Subcloned the gene encoding the cytoplasmic domain of Ire1 into the pET28a vector using the BamHI and NotI restriction sites. Construct codes for an N-terminal 6x-His tagged Ire1 cytoplasmic domain and includes its own stop codon. This yields the pET28a-ire1cyto-sp plasmid, pMD96. Similarly, inserted the cytoplasmic domain into pFastBacHTb using the BamHI and NotI restriction sites to yield pMD97. This plasmid also produces an N-terminal 6x-His tagged Ire1 cytoplasmic domain including its own stop codon.

**Purification of Recombinant *S. pombe* Ire1**

Ire1 cytoplasmic domain from *S. pombe* was expressed in SF21 insect cells from recombinant baculoviruses. Utilized the pFastBacHTb-Ire1cyto-sp plasmid pMD97. Generation of bacmids and viruses, as well as protein expression, was done according to the protocol outlined with the Bac-to-Bac® Baculovirus Expression System (Invitrogen). SF21 insect cells were grown in serum-free SF900-II SFM media at 28°C with shaking at 150 rpm. Bacmid DNA was transfected into cells and virus was allowed to amplify twice. Cells were then infected with virus for expression. Performed Western blot on cells from initial transfection to check protein expression. Took timepoints 25, 51, and 73 hours after infection to determine optimum time after transfection to harvest cells by Western blot. Used 73 hour sample to check solubility in 4 different buffers (Buffer 1: 100mM Tris pH8.0, 600mM NaCl, 2mM MgCl₂, 10% glycerol, 1% Triton X-100, COMPLETE protease inhibitor (Roche). Buffer 2: 100mM Tris pH8.0, 300mM NaCl, 2mM MgCl₂, 10% glycerol, 1% Triton X-100, COMPLETE protease inhibitor (Roche). Buffer 3: 100mM HEPES pH7.1, 600mM NaCl, 2mM MgCl₂, 10% glycerol, 1% Triton X-100,
Buffers were based on those used by Alexei Korennykh to purify the cytoplasmic domain of *S. cerevisiae* (Korennykh et al., 2009) and developed by Silke Nock and Han Li for the purification of human Ire1 (Li et al., 2010). To check solubility, frozen cells were resuspended in appropriate buffer. They were lysed by sonication and incubated in buffer at 4°C. Cell lysate was cleared by centrifugation at 100xg in the TLA 100.4 rotor. Supernatants were examined by Western blot.

The samples from the solubility test were next tested for binding to nickel resin. Tested HEPES pH7.1 and Tris-HCl pH8.0 in the 600mM NaCl high salt buffer. Added 5mM final concentration of imidazole to cleared lysates. Lysates were incubated with rotation at 4°C for 1 hour with the equivalent of 500µl of Nickel agarose slurry (Qiagen) that had been washed and equilibrated in Binding Buffer (50mM Tris pH8.0 or HEPES pH7.1, 600mM NaCl, 2mM MgCl$_2$, 10% glycerol, 1% Triton X-100, COMPLETE protease inhibitor (Roche), 5mM imidazole). After incubation, the flow through supernatant was removed from the beads. The beads were washed once in Binding Buffer, twice in 10mM Buffer (50mM Tris pH8.0 or HEPES pH7.1, 600mM NaCl, 2mM MgCl$_2$, 10% glycerol, 1% Triton X-100, COMPLETE protease inhibitor (Roche), 10mM imidazole) and twice in 20mM Buffer (50mM Tris pH8.0 or HEPES pH7.1, 600mM NaCl, 2mM MgCl$_2$, 10% glycerol, 1% Triton X-100, COMPLETE protease inhibitor (Roche), 20mM imidazole). Purified protein was eluted in 250mM Buffer (50mM Tris pH8.0 or HEPES pH7.1, 600mM NaCl, 2mM MgCl$_2$, 10% glycerol, 1% Triton X-100, COMPLETE protease inhibitor (Roche), 250mM imidazole) for 15 minutes at 4°C.
Purified 6xHIS-SpIre1cyto was examined on 10%, 10 well mini PROTEAN TGX gel (BioRad) and visualized by Coomassie staining.

For scaled up expression, harvested 400ml of SF21 cells at 73 hours post infection. Cell pellets were frozen at -80°C.

Results

Tagging of S. pombe Ire1

Ire1 protein was tagged with a 3x FLAG tag at the genomic locus of Ire1 in an initial effort to purify Ire1 directly from S. pombe. To preserve full function of the enzyme, three locations for tagging were selected and assayed for viability: the C terminus, the linker region and the so-called T loop portions of the enzyme. These locations were chosen based on locations that had been successfully tagged in S. cerevisiae Ire1. These strains were assayed for growth under UPR conditions by serial dilution on tunicamycin plates. The Ire1 tagged in the linker and T loop regions all showed mild growth defects by growth on plates under ER stress conditions, only the C-terminally tagged Ire1 grew at wildtype levels (data not shown). Additionally, Western blots against the 3x FLAG tag were performed to verify that the tag was in frame and properly expressed once integrated into cells. While all 3 versions of the tagged Ire1 protein were expressed and detected with an antibody directed against the 3x FLAG tag, the C-terminally tagged protein was expressed at a higher level than wildtype (Figure 1). Considering the growth phenotype and Western blot, further experiments were carried out using yMD82, the C-terminally tagged strain.
**In vitro Assay with Ire1-3xFLAG**

To determine if *S. pombe* Ire1 is directly cleaving the *Bip1* mRNA, C-terminally tagged *S. pombe* Ire1-3xFLAG was immunoprecipitated with anti-FLAG Dynabeads (Invitrogen). A similarly tagged *S. cerevisiae* Ire1 (Gardner and Walter, 2011) was grown in parallel as a control. We also wished to compare the cleavage abilities of *S. pombe* and *S. cerevisiae* Ire1 for both the *HAC1* mRNA and the *Bip1* mRNA. Western blot analysis showed that the immunoprecipitation of *S. pombe* Ire1 was not as efficient as that of *S. cerevisiae* Ire1 (Figure 2). It appears that the Ire1 protein in *S. pombe* was not expressed at the same level as its *S. cerevisiae* counterpart. However, there was Ire1 protein present on the beads indicating that the small amount of protein expressed in *S. pombe* was immunoprecipitated. As the protocol was continuous, we did not have the Western blot results prior to performing the *in vitro* cleavage assay. An *in vitro* cleavage assay was completed using both *S. pombe* and *S. cerevisiae* Ire1 bound to Dynabeads. Each protein was incubated with *in vitro* transcribed radiolabeled 3’UTR of *Bip1* mRNA or *HAC1* U508 RNA, separated on a polyacrylamide-TBE-Urea gel and exposed to film (Figure 3). This preliminary cleavage assay was not successful and the control reaction between *S. cerevisiae* Ire1 and the *HAC1* U508 RNA failed to cleave.

**Expression of S. pombe Ire1 in E. coli**

The *S. cerevisiae* Ire1 cytoplasmic domain has been successfully expressed and purified from *E. coli* (Korenykh et al., 2009; Sidrauski and Walter, 1997). An alignment between the *S. cerevisiae* and the *S. pombe* proteins was performed and the portion of the gene encoding *S. pombe* Ire1 homologous to the *S. cerevisiae* Ire1 cytoplasmic domain
previously purified and crystallized in our lab was cloned (Korennykh et al., 2009). Initially, attempts were made to express the *S. pombe* cytoplasmic domain in *E. coli*. First it was expressed as a GST fusion protein, as had been done in *S. cerevisiae* (Korennykh et al., 2009). However, this construct was difficult to clone and failed to express in a variety of *E. coli* strain backgrounds, possibly indicating toxicity to *E. coli*. The construct was then cloned into a pET vector containing the more highly repressed T7 promoter, which is better at repressing toxic protein products. The protein still failed to express when a variety of strains and conditions were attempted (data not shown). Overall, the *S. pombe* Ire1 cytoplasmic domain appears to be toxic to *E. coli* cells.

**Purification of Recombinant *S. pombe* Ire1 cytoplasmic domain**

Previous results from our lab have shown that cytosolic domain of human Ire1 is difficult to express in *E. coli* cells and may be toxic. To overcome this problem, the human protein was expressed and successfully purified from insect cells (Li et al., 2010). As the expression of *S. pombe* Ire1 protein also proved to be difficult from *E. coli* cells, the construct was cloned into a attempts were then made to express and purify the recombinant protein from insect cells. Initial experiments show that *S. pombe* Ire1 cytoplasmic domain is indeed expressed well in SF21 insect cells (Figure 4). A timecourse of expression revealed that the protein is expressed well at all timepoints analyzed (Figure 5). Additionally, the protein was soluble and able to be crudely purified by the 6xHIS tag over nickel beads (Figure 6).
*S. pombe* Ire1 in *S. cerevisiae*

While the HAC1 mRNA is the only known substrate of the *S. cerevisiae* Ire1 enzyme, it is able to cleave the human Ire1 substrate, Xbp1, *in vitro* (Korennykh et al., 2009; Niwa et al., 2005). Likewise, elements of the HAC1 mRNA can be cleaved by mammalian Ire1 and when expressed in mammalian cells, HAC1 is faithfully spliced when the UPR is induced (Niwa et al., 1999; Tirasophon et al., 1998). In a similar fashion, we wished to examine if *S. pombe* Ire1 was capable of recognizing and cleaving the HAC1 splice sites. To this end, we transformed a plasmid expressing an intronless version of *S. pombe* Ire1 into a budding yeast strain carrying an integrated copy of the HAC1 splicing reporter (Pincus, 2010). The UPR was induced and splicing was measured by flow cytometry. In this initial assay, it appeared that *S. pombe* Ire1 did not cleave the HAC1 splicing reporter (data not shown). However, Ire1 was being expressed from its own *S. pombe* promoter in *S. cerevisiae* cells. As Ire1 was not tagged, we were not able to confirm that Ire1 was successfully expressed in the budding yeast cells.

**Discussion**

The work described here is the preliminary effort to further characterize the *S. pombe* Ire1 protein. Initially, we wanted to show that Ire1 was responsible for the direct cleavage of the Bip1 mRNA. We started with efforts to crudely purify the full-length protein via a C-terminal 3x FLAG tag and directly use this protein in *in vitro* RNA cleavage assays. This proved to be a complicated and involved protocol and initial *in vitro* experiments with immunoprecipitated Ire1-3x FLAG failed (Figure 3). With the ultimate goal of obtaining purified Ire1 cytoplasmic domain for *in vitro* assays, we
attempted to purify the cytoplasmic domain of Ire1 from *E. coli* cells and SF21 insect cells. While Ire1 appeared to produce a toxic protein in *E. coli* cells, we were able to successfully express and purify *S. pombe* Ire1 from SF21 insect cells (Figure 6). The initial test expressions and purification in insect cells looks promising, but does require further optimization and scaling up in order to produce enough protein for *in vitro* RNA cleavage assays.

The 3x FLAG tagged Ire1 did not prove to be suitable for purification from *S. pombe* for the purpose of subsequent *in vitro* RNA cleavage assays. However, this yeast strain could prove to be very useful as a tool for other studies of the UPR in *S. pombe*. It has been verified to complement for wildtype Ire1 during ER stress. Thus, having a tagged version of Ire1 allows for simple detection by Western blot or visualization with immunofluorescence. It will also aid in the study of *S. pombe* function in other cells as expression of fission yeast Ire1 can be verified. In addition to the 3xFLAG tagged protein, a construct for C-terminally Venus tagged Ire1 was generated and can be found as pMD90. This construct was made and never tested.

This work is the beginning of what should prove to be a fruitful journey into studying the mechanism of fission yeast Ire1. Purified *S. pombe* Ire1 can be used for *in vitro* assays to characterize the consensus cleavage site for Ire1 as was performed in budding yeast (Gonzalez et al., 1999). We can challenge the *S. pombe* Ire1 protein to test the limits of its tolerance for different RNA sequences to define the minimal sequence and secondary structure requirements for *S. pombe* Ire1 mediated RNA cleavage. The best substrates to begin with are those that have already been established in assays with *S. cerevisiae*, such as various *in vitro* transcribed regions of *HAC1* (Gonzalez et al., 1999;
Sidrauski and Walter, 1997) or the minimal hairpin loops (Gonzalez et al., 1999; Korenykh et al., 2008). We could then directly test regions of Bip1 mRNA and other downregulated targets like Gas2 mRNA (Chapter 2). Furthermore, a series of careful in vitro assays utilizing purified Ire1 and RNA molecules containing various consensus sequences will allow us to conclusively show that the cytosolic domains of Ire1 are directly cleaving Bip1 and RIDD targets in fission yeast.

Beyond these initial goals, purified cytoplasmic S. pombe Ire1 can also be used for structural analysis. Crystallization of the protein will allow for further comparison with mammalian and budding yeast Ire1 molecules. Such comparisons of the cytoplasmic domain may highlight the integral differences that allow S. pombe Ire1 to have repurposed itself for RIDD-like cleavage of a subset of mRNAs, while budding yeast Ire1 recognizes only one substrate (Niwa et al., 2005). Additionally, it may shed light on how mammalian Ire1 has evolved to switch between both mechanisms: cleavage of its substrate, Xbp1, and RIDD.

This work only briefly touched on the idea of testing S. pombe Ire1 function in S. cerevisiae. Here, we tried to place the fission yeast Ire1 into budding yeast and examine its ability, as the only copy of Ire1, to splice the HAC1 splicing reporter as measured by flow cytometry. When the UPR was induced, the S. pombe Ire1 failed to splice the HAC1 reporter. There are many reasons for this and the failure of this experiment does not mean that this is not a promising line of investigation. The intron was removed from Ire1 for fear that S. cerevisiae would be unable to splice the Ire1 mRNA. However, the protein remained under the control of its own S. pombe promoter and it is unclear if this promoter would function in S. cerevisiae. At the time, Ire1 was not tagged in fission yeast.
Therefore, we had no means of confirming that the protein was actually expressed in *S. cerevisiae*. As a first pass, it would be beneficial to transform the fission yeast Ire-3xFLAG construct into *S. cerevisiae* and determine if the Ire1 protein is expressed by Western blot. If not, a follow up experiment would be to place the tagged Ire1 under the control of a budding yeast promoter. There are many permutations of such experiments to test the interchangeability of fission yeast and budding yeast Ire1 that one could envision. These experiments could elucidate other features of Ire1 that are conserved or differ between these two yeast species.
### Table 1. Plasmids

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene Involved</th>
<th>Vector Backbone</th>
<th>Marker</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMD50</td>
<td><em>S. pombe</em> ire1</td>
<td>pJK210</td>
<td><em>ura4</em></td>
<td>this study</td>
</tr>
<tr>
<td>pMD54</td>
<td><em>S. pombe</em> ire1</td>
<td>pRS316</td>
<td><em>URA3</em></td>
<td>this study</td>
</tr>
<tr>
<td>pMD76</td>
<td><em>S. pombe</em> ire1-3xFLAG (C-term)</td>
<td>pRS316</td>
<td><em>URA3</em></td>
<td>this study</td>
</tr>
<tr>
<td>pMD77</td>
<td><em>S. pombe</em> ire1-3xFLAG (T loop)</td>
<td>pRS316</td>
<td><em>URA3</em></td>
<td>this study</td>
</tr>
<tr>
<td>pMD78</td>
<td><em>S. pombe</em> ire1-3xFLAG (linker)</td>
<td>pRS316</td>
<td><em>URA3</em></td>
<td>this study</td>
</tr>
<tr>
<td>pMD79</td>
<td><em>S. pombe</em> ire1-3xFLAG (C-term)</td>
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<td>pJK210</td>
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<td>this study</td>
</tr>
<tr>
<td>pMD81</td>
<td><em>S. pombe</em> ire1-3xFLAG (linker)</td>
<td>pJK210</td>
<td><em>ura4</em></td>
<td>this study</td>
</tr>
<tr>
<td>pMD84</td>
<td><em>S. pombe</em> ire1Δintron</td>
<td>pRS316</td>
<td><em>URA3</em></td>
<td>this study</td>
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<tr>
<td>pMD90</td>
<td><em>S. pombe</em> ire1-Venus (C-term)</td>
<td>pJK210</td>
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<td>pMD95</td>
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<td>pET28a</td>
<td><em>kan</em></td>
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<tr>
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<td><em>S. pombe GST</em>-ire1-cyto</td>
<td>pFastBacHTb</td>
<td><em>amp</em></td>
<td>this study</td>
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### Table 2. Yeast Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>W303-1B</td>
<td><em>S. cerevisiae</em>; MAT α; ade2-1; trp1-1; can1-100; leu2-3,112; his3-11,15; ura3-1; psi+ (from ATCC frozen as yMD1)</td>
</tr>
<tr>
<td>yMD76</td>
<td><em>S. pombe</em>, h+, ade6-M210, ura4-D18, leu1-32</td>
</tr>
<tr>
<td>yMD82</td>
<td><em>S. pombe</em>, h+, ade6-M210, ura4-D18, leu1-32, ire1-3xflag(C-term)::IRE1</td>
</tr>
<tr>
<td>yMD83</td>
<td><em>S. pombe</em>, h+, ade6-M210, ura4-D18, leu1-32, ire1-3xflag(T-loop)::IRE1</td>
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<tr>
<td>yMD84</td>
<td><em>S. pombe</em>, h+, ade6-M210, ura4-D18, leu1-32, ire1-3xflag(Linker)::IRE1</td>
</tr>
<tr>
<td>Name</td>
<td>5'-3' Sequence</td>
</tr>
<tr>
<td>-------</td>
<td>-------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>oMD291</td>
<td>CGGggtaccGAACATATGACTTATCCTCAG</td>
</tr>
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<td>oMD292</td>
<td>GGCgagctcGTAATGTATTTACTCCATTACG</td>
</tr>
<tr>
<td>oMD329</td>
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</tr>
<tr>
<td>oMD446</td>
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</tr>
<tr>
<td>oMD447</td>
<td>AAGGAAAAAgcgccgcTCAATACTTCTAGATAACGT TTATGAC</td>
</tr>
</tbody>
</table>
Figure 1. 3xFLAG Tagged Ire1 is Expressed in *S. pombe*

*Streptomyces pombe* strains carrying integrated copies various 3xFLAG tagged *S. pombe* Ire1 proteins were subject to Western blot and protein was detected with an anti-FLAG antibody (Sigma). In all cases, Ire1 was expressed in these cells and can be seen at the expected size of approximately 130kDa. Lane 1 contains wildtype yMD76, lane 2 contains the C-terminally tagged protein from yMD82, lane 3 contained the T-loop tagged protein from yMD83, and lane 4 contains the linker tagged Ire1 from yMD84. The C-terminally tagged protein shows a higher level of expression than the other two proteins.
Figure 1.
Figure 2. Immunoprecipitation of Ire1-3xFLAG

C-terminally tagged Ire1 was immunoprecipitated by α-FLAG antibody conjugated to Dynabeads from both *S. pombe* and *S. cerevisiae* cells after induction with 2mM DTT. Fractions were collected from the total, pellet, unbound (UB) and bead portions of the immunoprecipitation and subjected to Western blotting with antibody against the FLAG tag. Ire1 from both strains was purified. However, much protein was unsolubilized and lost in the pellet fraction and the *S. pombe* protein had lower levels of expression than the *S. cerevisiae* protein. The expected location of the full length Ire1 protein is indicated with an arrow.
Figure 2.
Figure 3. *In vitro* Ire1 Cleavage Assay

Immunoprecipitated material from the bead fraction of the 3xFLAG tagged immunoprecipitation of Ire1, shown in Figure 2, was incubated with body labeled *in vitro* transcribed *HAC1* or *Bip1* mRNA probe in an *in vitro* cleavage assay. Probe alone was run in lanes 1 and 2, marked H for *HAC1* and B for *Bip1*. Lane 3 contains *HAC1* probe incubated with *S. cerevisiae* Ire1, lane 4 is *HAC1* probe incubated with *S. pombe* Ire1, lane 5 contains *Bip1* mRNA incubated with *S. cerevisiae* Ire1 and lane 6 is *Bip1* probe with *S. pombe* Ire1. Cleavage was not seen in any combination of RNA and enzyme.
Figure 3.
**Figure 4. The *S. pombe* Ire1 Cytoplasmic Domain is Expressed in SF21 Insect Cells**

Four different clones of the *S. pombe* 6xHIS tagged Ire1-cytoplasmic domain were transfected and expressed in SF21 Insect Cells. Cells were collected and lysed in protein gel loading buffer and subject to Western blot analysis with anti-HIS antibody (AbCam). The expected size of the Ire cytoplasmic domain is indicated with an arrow. In all clones, Ire1 was expressed well and no expression was seen in the negative controls, untransfected cells and cells transfected with empty bacmid. The band indicated by an arrow is an unidentified protein. It is not seen in the controls and, therefore, is not a background band of the antibody. It may represent a cleavage product of Ire1.
Figure 4.
Figure 5. Timecourse of Ire1 Expression in SF21 Insect Cells

SF21 insect cells were collected at 25 hours, 51 hours and 73 hours post transfection for all four clones of 6xHIS-Ire1 cytoplasmic domain. They were subject to Western blotting with anti-HIS antibody (AbCam) to determine the ideal time post transfection to harvest cells in order to optimize protein expression and yield. Negative controls were untransfected cells and cells transfected with empty bacmid. In all cases protein production was highest at the 73 hour timepoint.
Figure 5.
Figure 6. Purification of *S. pombe* 6xHIS-Ire1 Cytoplasmic Domain from SF21 Cells

The 6xHIS-Ire1 cytoplasmic domain was crudely purified from SF21 insect cells 73 hours post transfection with nickel resin (Qiagen). Two buffers were tested as indicated. The total protein (T), unbound flow through (UB), first wash (W1), second wash (W2), three eluted fractions (E1, E2, E3) and the beads (B) were separated by PAGE and then Coomassie stained. Both buffers appeared to function equally. The protein readily bound to the nickel resin. However, in both cases, the eluates were quite dirty and required more stringent washes.
**Figure 6.**

<table>
<thead>
<tr>
<th>Buffer 1: HEPES</th>
<th>Buffer 2: Tris</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>UB</td>
</tr>
<tr>
<td>T</td>
<td>UB</td>
</tr>
</tbody>
</table>

Buffer 1:
- 600mM NaCl
- 100mM HEPES pH 7.4
- 2mM MgCl₂
- 10% glycerol
- 1% Triton

Buffer 2:
- 600mM NaCl
- 100mM Tris pH 8.0
- 2mM MgCl₂
- 10% glycerol
- 1% Triton
References


Chapter 4

Mutation in Ski2: Identification of a Dominant Suppressor of \textit{trl1-100}
Introduction

The budding yeast tRNA ligase, Trl1, is an essential protein with three catalytic activities. Each catalytic activity resides in distinct domains of Trl1 that can be expressed and function as separate peptides (Sawaya et al., 2003). Trl1 is composed of an N terminal adenylyltransferase domain, a kinase domain and a cyclic phosphodiesterase.

tRNA ligase was first discovered to have a role in the UPR through a genetic screen in the yeast *S. cerevisiae* looking for essential components of the UPR (Sidrauski et al., 1996). A single allele of *TRL1* was isolated, *trl1-100*, and was shown to be defective in *HAC1* splicing *in vivo*, but was still fully functional in its ability to splice tRNA. The mutation was identified as H148Y and is in a single conserved histidine residue in the adenylyltransferase domain. When the UPR is activated in *trl1-100* cells, Ire1 cleaves the *HAC1* mRNA, but the defective ligase is unable to ligate the resulting fragments. The *HAC1* mRNA fragments are degraded and thus the cell is unable to survive ER stress. However, despite defects *in vivo*, the mutant ligase has been shown to successfully ligate *HAC1* mRNA *in vitro* (Gonzalez, 2003). The mechanism of the *trl1-100* defect *in vivo* is still not understood.

In an effort to understand the role of Trl1 in the UPR, a screen looking for suppressors of the *trl1-100* mutation was carried out in our lab (Gonzalez, 2003). This screen isolated 70 suppressor mutations based on their ability to rescue the *trl1-100* growth defect on UPR inducing plates. Termed Suppressors of *rlg1-100* (SOR) mutations, these strains were then classified further into three groups by their *HAC1* splicing phenotypes assayed by Northern blotting. The first group, SOR1, included 10

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1 Trl1 is also known as Rlg1.
dominant mutations that restored \textit{HAC1} splicing. Sequencing of the \textit{TRL1} gene revealed that these suppressors all contained secondary mutations within the \textit{trl1} gene (Rubio, 2010). However, none reverted \textit{trl1-100} back to wildtype \textit{TRL1}. The second group of suppressors, SOR2, consisted of 9 mutations, 7 dominant and 2 recessive, that restored growth on tunicamycin plates, but did not reestablish \textit{HAC1} splicing (Table 1). Northern blot analysis further revealed an increase in the \textit{HAC1} mRNA fragments from the 5’ exon and 5’exon-intron. Genetic analysis of these strains showed that all carried extragenic suppressors of \textit{trl1-100} (Gonzalez, 2003). The third class of suppressors, SOR3, made up the majority of the isolated suppressor mutations. While these strains allowed for restored growth under ER stress conditions, the \textit{HAC1} splicing phenotype of these strains was unchanged. We hypothesize that these mutations reside in genes that aid in survival downstream of Hac1 production by possibly increasing ERAD or folding machinery or decreasing translocation into the ER. This class of mutations was not characterized further.

Of the three classes of SOR mutations, the most effort has been exerted trying to characterize the SOR2 extragenic suppressors of \textit{trl1-100}. The two recessive mutations were initially chosen for further characterization. It was shown that the stabilized \textit{HAC1} 5’ exon fragment is translated and the resulting truncated transcription factor is responsible for their ability to grown under ER stress conditions (Gonzalez, 2003). Moreover, the \textit{HAC1} 5’ splice site is required for the observed growth phenotype and the production of truncated Hac1 protein (Gonzalez, 2003). When the 5’ splice site is mutated, translational repression by the \textit{HAC1} intron is not relieved and \textit{HAC1} cannot be translated. In the SOR2 mutants, the stabilized \textit{HAC1} 5’ exon no longer has a stop codon
or polyA tail and should be subject to rapid RNA decay. Therefore, it was hypothesized that SOR2 mutations are likely mutations in the RNA degradation machinery. A plasmid based assay was utilized to confirm that the recessive mutant strains are indeed defective for nonstop mediated decay (Gonzalez, 2003; van Hoof et al., 2002).

The efforts to identify the mutations behind the SOR2 phenotypes by standard genetic means failed; therefore a whole genome sequencing strategy was developed. Genomic DNA from two of the SOR2 strains (PWy707 and PWy712) was subjected to a unique sequencing protocol designed to control for single nucleotide polymorphisms that would obstruct the identification of causal point mutations (Rubio, 2010). Using high throughput genome wide sequencing, several point mutations were identified in strain PWy712\(^2\). Among these mutations was a mutation in the *SKI2* gene (G1361A)\(^3\) causing an histidine substitution at arginine 454 in the Ski2 protein (Rubio, 2010).

Ski2 is a DExD-box helicase and an integral component the yeast 3’-5’ cytoplasmic mRNA degradation pathway (Anderson and Parker, 1998). It has been shown to be in complex with Ski3 and Ski8 to form the heterotrimeric helicase Ski2/Ski3/Ski8 complex that participates primes mRNAs for 3’-5’ degradation (Brown et al., 2000; Wang et al., 2005). The Ski2/Ski3/Ski8 complex has also been shown to interact with the Ski7 GTPase, which is also required for 3’-5’ degradation (Araki et al., 2001). It is thought that Ski7 bridges the interaction between the Ski complex and the exosome. The Ski2 helicase shares substantial sequence identity with its nuclear counterpart, the helicase Mtr4. The structures of both proteins have been determined by crystallization (Halbach et al., 2012; Weir et al., 2010). While Ski2 is known to play a

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\(^2\) Stated incorrectly as PWy707 in Rubio, 2010.

\(^3\) Stated incorrectly as G1360A in Rubio, 2010.
crucial role in 3’-5’ mRNA decay, little is known about the mechanism of its action. However, it has been proposed that the presence of helicases to prime exosome degradation could function to recognize mRNA substrates, prime substrates by unwinding secondary structure and removing RNA binding proteins. In this way the helicases may serve to present suitable single stranded RNA to the exosome for degradation (Houseley and Tollervey, 2009).

The role of Ski2 in RNA degradation makes it an excellent candidate as the gene likely responsible for the observed suppressor phenotype. However, this was never verified as the mutation causing the trl1-100 suppressor phenotype. In Chapter 4 of this thesis, we focus on the continued efforts to identify extragenic dominant SOR2 mutations. We show the construction of a dominant genomic library and efforts to use genetic complementation to identify the mutation found in PWy707. Additionally, we show that the G1361 mutation in SKI2 is responsible for the stabilization of HAC1 exons in trl1-100 cells.

**Materials and Methods**

All yeast strains used in this study can be found in Table 2. The oligonucleotide primer sequences used for PCR amplification and cloning can be found in Table 3. Sequencing primer sequences can are listed in Table 4.

**SOR2 dominant library**

To construct the genomic DNA library, total intact yeast genomic DNA was isolated from a SOR2 strain (PWy707). This DNA underwent a partial digestion with
Sau3Al (NEB) for 15 minutes at 37°C and phenol-chloroform extracted. Digested DNA was separated over a 5-20% potassium acetate gradient made on a Biocomp Gradient Master and centrifuged for 50,000 rpm for 1 hours in an SW55 rotor at room temperature. A total of 25 approximately 200µl fractions were isolated and ethanol precipitated. A small amount of each fraction was visualized on a 0.5% agarose gel. Fractions containing DNA fragments between 7 and 10 KB were used for ligation. The yeast cloning vector pRS315 was digested with BamHI carefully to avoid STAR activity of the enzyme and then phosphatase treated with Antarctic Phosphatase (NEB). Digested genomic DNA inserts were ligated to BamHI digested pRS315 vector DNA with T4 DNA ligase (NEB). The ligation reaction was transformed into DH5α E. coli cells. Transformed cells were plated for single colonies on LB plates containing ampicillin. Cells were collected from the surface of the plates and plasmid DNA library was purified using Midi prep DNA kits (Qiagen).

The SOR2 genomic DNA library was transformed into a trl1-100 strain (PWy610) using a standard high efficiency lithium acetate transformation protocol. Transformants were selected on SD–leu plates. For selection of the UPR suppressor phenotype, colonies were replica plated onto SD–leu plates containing tunicamycin. Colonies that were able to grow were restreaked onto fresh SD–leu plus tunicamycin plates to confirm the growth phenotype. Plasmid DNA was isolated from these strains and sequenced.
Verification of Mutation in Original Strain

PCR amplified the *SKI2* gene with *Pfu* TURBO (Strategene) from genomic DNA isolated from the following yeast strains: PWy612, PWy707 and PWy712. Used the forward oligonucleotide primer oMD367 and reverse oligonucleotide primer oMD368, which added *SacII* and *XbaI* sites respectively, shown in lower case, flanking the *SKI2* gene. Gel purified PCR product and sent product for sequencing using oMD347.

Yeast Strain Construction

To remake a strain containing the *ski2* gene mutation identified in one of the SOR2 strains, the *SKI2* genes were PCR amplified from genomic DNA isolated from PWy612 and PWy712 with primers oMD367 and oMD368. Gel purified PCR product was cloned into the integrating yeast vector pRS306 using *SacII* and *XbaI* sites. Sequenced plasmids with oligonucleotide primers oMD347-oMD355. The new strain was constructed using a loop-in, loop-out technique. pRS306-*SKI2* and pRS306-*ski2*-1 were linearized with *SphI* at 37°C for 2 hours and transformed into PWy610 using a standard *S. cerevisiae* transformation protocol. Selected for positive transformants on SD–ura dropout media plates. Grew positive colonies overnight in YPD rich media liquid culture at 30°C to allow for loop out of URA3 marker. Colonies were plated on 5-Fluoroorotic Acid (5-FOA) plates to select for cells that had successfully lost the URA3 marker.

To test dominance and retest the previous work done with a recessive SOR2 mutant, the mutant *ski2* gene was subcloned out of pRS306 with *SacII* and *XbaI* and placed into the yeast centromeric vector pRS315. Using a standard yeast lithium acetate transformation protocol, pRS315-*ski2*(G1361A) was then transformed into the following
yeast strains: PWy764, PWy765, PWy768, PWy769, PWy771, and PWy772.
Transformants were selected for on SD–leu plates and all further work with these strains was performed using SD–leu dropout media to maintain plasmid selection.

**Northern blot**

Cells were grown overnight in YPD rich media at 30°C, diluted to an OD$_{600}$ of 0.05 and grown to an OD$_{600}$ of 0.2. The UPR was induced by the addition of 8mM DTT for 40 minutes at 30°C. Harvested cells by centrifugation and snap froze cell pellets in liquid nitrogen.

Total RNA was isolated from yeast cells using a standard hot phenol-chloroform extraction (Ruegsegger et al., 2001). Northern blot analysis was carried out using 12µg of total RNA separated on a 1.5% (w/v) denaturing formaldehyde agarose gel. RNA was transferred to a nitrocellulose membrane (GE Water and Press Technologies) using capillary transfer overnight and UV crosslinked to the membrane. The HAC1 mRNA was detected using $^{32}$P radioactive labeled 500bp probe directed to the 5’ exon of the transcript (Cox and Walter, 1996) and visualized by exposure to a phosphorimaging screen scanned on a Typhoon (GE).

**Plate Growth Assay**

Grew strains overnight in liquid YPD rich media at 30°C. Diluted overnight cultures to OD$_{600}$ of 0.05 into fresh liquid YPD media. Allowed to grow for 3 hours at 30°C until OD$_{600}$ reached approximately 0.2. Diluted strains to an OD$_{600}$ of 0.50 in a sterile 96 well plate. Made six 1:5 dilutions. Spotted 2µl of each sample using a multi-
channel pipetman onto each of the growth media in plates. Grew plates for 2-3 days at 30ºC.

**Sequencing of Candidate Genes in SOR2 Strains**

Genes involved in cellular mRNA degradation pathways were identified as likely candidates for the suppressor phenotype. They were as follows: *SKI2, SKI3, SKI7, SKI8*, and *CSL4*. Genes were amplified by PCR using the *Pfu* TURBO enzyme (Strategene) and the appropriate oligonucleotide primer pair (Table 3) from each of the SOR2 strains shown in Table 1. The resulting PCR products were gel purified and each gene was fully sequenced using oligonucleotide primers directed against the gene sequence (Table 4).

**Results**

*A SOR2 Dominant Genomic DNA Library*

In efforts to further understand the role of tRNA ligase in the UPR, a genetic screen for suppressors of the *trl1-100* allele was conducted. This screen selected for mutations that could rescue the growth defect of *trl1-100* cells when plated on UPR inducing media. Of the suppressor mutations isolated, nine mutations were classified as SOR2 for their ability to stabilize the Ire1 cleaved fragments of *HAC1* (Table 1). Two of these mutations were recessive and had been the focus of previous efforts to clone and characterize the mutations (Gonzalez, 2003). Attempts were made to identify the recessive mutation by standard complementation with a yeast genomic library. However, these efforts proved to be futile. In this study the focus was shifted away from the recessive mutations and onto the strains harboring dominant mutations.
To identify and clone dominant mutations, it is typical to create a genomic library from genomic DNA isolated from the dominant strain. This genomic DNA library is then transformed back into the original screen strain. The strains are then tested for the desired dominant phenotype. Many attempts had been made to create such a library for the dominant SOR2 strains, yet none had been successful thus far. Here, renewed efforts were made to construct a genomic DNA library from one of the dominant SOR2 strains, PWy707. We successfully completed a genomic library for PWy707 and transformed it into a trl1-100 strain (PWy610). Transformants were plated on UPR inducing plates and colonies that were able to grow were re-streaked onto fresh plates to confirm the growth phenotype. Plasmids were rescued from strains that were able to grow on UPR inducing plates and sequenced to determine the identity of the genomic fragment. Two genes repeatedly appeared among these fragments, HAC1 and SUP53 (the gene encoding the leucine tRNA). However, when these gene inserts were sequenced, we found that they were wildtype copies of the genes. Extra copies of these genes are able to rescue the trl1-100 UPR specific growth defect by unknown mechanisms. Ultimately, despite the successful construction of a genomic library, we were unable to identify the SOR2 mutation in PWy707 using this method.

**Identification and Characterization of a ski2 Mutation**

Despite difficulty identifying the dominant suppressor phenotypes through standard genetic techniques, an attempt to sequence the entire genome using high throughput sequencing techniques was ultimately successful (Rubio, 2010). A candidate point mutation was identified in the SKI2 gene encoding for a DExD-box helicase that is
a component of the cytoplasmic 3’-5’ RNA degradation pathway (Anderson and Parker, 1998). Two of the dominant SOR2 strains were fully sequenced, PWy707 and PWy712, and the mutation was identified in one of these strains. Initially there was some confusion as to which strain harbored the ski2(G1361A) mutation. The SKI2 genes were amplified from the original trl1-100 screen strain (PWy610) and from both SOR2 strains. The genes were sequenced and it was determined that the PWy712 strain harbored the mutant ski2(G1361A) gene.

To verify that the ski2(G1361A) mutant was responsible for the rescue of the trl1-100 UPR growth phenotype, we initially tested the mutation on a plasmid. A centromeric shuttle vector containing ski2(G1361A) was transformed into a trl1-100, Δski2 strain. Growth of this strain was then tested by serial dilution onto tunicamycin containing media (Figure 1). The plasmid harboring the ski2(G1361A) was able to rescue the trl1-100 UPR growth phenotype, but did not restore growth to the same level as the original SOR2 mutant strain.

For a next level of certainty we remade the mutant strain. The genomic copy of SKI2 was mutated to ski(G1361A) in the original trl1-100 screen strain (PWy610) using a loop in loop out technique to create yMD90. Colony PCR and subsequent sequencing verified the location and presence of the mutation at the SKI2 locus. The suppressor phenotypes were then retested with this fresh strain. The UPR growth phenotype was tested on plates containing the UPR inducing agent tunicamycin. Cells were grown and plated in serial dilution on the media (Figure 2). The ski2(G1361A) mutation was able to rescue the growth of trl1-100 on UPR plates. However, the growth was not rescued to the full extent seen in the original PWy712 strain and yMD90 appeared sick even on control
plates. Additionally, the UPR was induced and \textit{HAC1} mRNA was analyzed by Northern blot (Figure 3). The splicing phenotype of the new strain harboring the \textit{ski2(G1361A)} mutation was identical to the SOR2 PWy712 strain and was able to stabilize the 5’exon and 5’exon-intron of \textit{HAC1}.

To further characterize the \textit{ski2(G1361A)} phenotype, we began to repeat experiments that had been done previously for the SOR2 recessive mutations (Gonzalez, 2003). The hypothesis is that the SOR2 mutation stabilizes the \textit{HAC1} cleaved fragments. Therefore, we propose that the SOR2 ability to grow on UPR inducing media is the result of translation from one of the stabilized fragments to yield a truncated form of the Hac1 transcription factor. Translation of the \textit{HAC1} mRNA is blocked by basepairing between an inhibitory intron and the 5’UTR that likely blocks translation initiation (Rüegsegger et al., 2001). When Ire1 disrupts the basepairing interaction through splicing of the intron, the translational block is released. In SOR2 mutants, Ire1 cleavage at the 5’ splice site and subsequent stabilization of the 5’ exon would also relieve the translational repression. Mutations in the RNA degradation pathway, such as the \textit{ski2(G1361A)} mutation, would likely stabilize mRNA fragments like the \textit{HAC1} 5’ exon. This model would suggest that mutating the 5’ splice site, but not the 3’ splice site, would no longer allow for production of a truncated Hac1 and thus these cells would no longer grow under UPR conditions. To test this hypothesis, we inserted the \textit{ski2(G1361A)} gene into an array of strains containing an HA tagged Hac1 with either wildtype sequence or mutations in either the 5’ or 3’ splice sites. This was done in either the \textit{TRL1} or \textit{trl1-100} background. A centromeric shuttle vector was constructed containing the \textit{ski2(G1361A)} gene. This vector was transformed into the HA-Hac1 strains PWy764, PWy765, PWy768, PWy769, PWy771.
and PWy772. The wildtype copy of SKI2 was left in these strains because the mutation from the SOR2 strain containing ski2(G1361A), PWy712, was previously shown to be dominant (Gonzalez, 2003). These strains were assayed for growth by serial dilution on tunicamycin containing plates (Figure 4). Surprisingly, the control strain containing a plasmid borne copy of ski2(G1361A) did not rescue the growth in the control trl1-100 strain when expressed over wildtype SKI2. As this plasmid has rescued the UPR specific growth defect of trl1-100 in a ∆ski2 strain and when integrated as the only copy of the gene, these results indicate that the ski2(G1361A) mutation may be sensitive to expression levels or may not be dominant. The results from the remainder of this experiment are not interpretable because the controls did not work.

**Sequencing of Candidate Genes in SOR2 Strains**

We have shown that the SOR2 mutant strains likely harbor mutations in the RNA degradation machinery. Therefore, rather than sequence the remaining SOR2 mutations genome wide, we pursued a brute force sequencing method to sequence genes encoding for components of the RNA degradation pathway. We hoped that by sequencing candidate genes we would be able to identify a few more of the SOR2 mutations. In each of the six remaining unidentified SOR2 mutants that could be found in the Walter lab yeast collection, the following genes were sequenced: SKI2, SKI3, SKI7, SKI8, and CSL4. Unfortunately, the five genes we chose to sequence did not contain any mutations (data not shown). Therefore, the remaining SOR2 mutations have yet to be identified.
Discussion

The path towards identifying the SOR2 mutations has been long. It began with failed attempts to complement the recessive SOR2 mutations with a genomic library and continued with difficulties constructing genomic libraries from the dominant strains. Once the obstacle of constructing a dominant genomic DNA library had been surmounted, the library again failed to identify the causal mutation. We have since sequenced the entire genome of two of the dominant strains and identified a very promising candidate in the ski2(G1361A) mutation. However, this mutation does not behave exactly as we would predict.

Retesting of the ski2(G1361A) mutation in trl1-100 cells definitively shows that this mutation is responsible for the HAC1 splicing phenotype and stabilizes HAC1 mRNA fragments (Figure 3). However, the repetition of the UPR growth phenotype on plates is less clear. The mutation does appear to rescue the growth of trl1-100 on UPR plates. However, the cells are much sicker than their PWy712 counterpart and do not rescue growth to the same degree as the originally isolated SOR2 strains (Figure 2). This could indicate that the original PWy712 strain contains a secondary mutation. However, this is not likely because the original strains were backcrossed prior to characterization (Gonzalez, 2003). Additionally, attempts to further characterize the SOR2 defect using the ski2(G1361A) mutation on a centromeric plasmid over the wildtype SKI2 gene are not readily interpretable. When expressed on a plasmid, the ski2(G1361A) mutation failed to rescue the UPR growth defect of the control trl1-100 strain with wildtype SKI2 present (Figure 4). This same plasmid was able to rescue trl1-100 growth on UPR plates when in a Δski2 background (Figure 1). Together, this indicates that the ski2(G1361A) mutation
may be very sensitive to expression levels or may not be dominant as was previously reported (Gonzalez, 2003). The dominance of this mutation should be checked by crossing yMD90 to wildtype. If it is indeed dominant, then the various HA-\textit{HAC1} strains should be remade with the \textit{ski2(G1361A)} mutation integrated into the wildtype genomic locus of the gene.

One of the exciting features of these mutations is the promise that they are dominant. In this way, they could be used as a tool to stabilize RNA within cells by simply transforming in a copy of the mutant gene on a plasmid. However, the most recent results with \textit{ski2(G1361A)} puts the dominance of the SOR2 mutations into question. If work with these strains is to be pursued in the future, sound first steps would be to backcross the strains again to ensure that only one gene is responsible for the observed SOR2 phenotype and retest for dominance in all of the strains. As technology advances and high throughput sequencing techniques become more affordable, sequencing of the remaining SOR2 mutations may prove to be the easiest way to identify them. If some of the strains are not dominant, this could easily explain why we were not able to clone the mutation with our genomic DNA library.

The original \textit{trl1-100} suppressor screen was designed to provide insight into the role of tRNA ligase in the UPR and further understand the \textit{trl1-100} UPR specific defect. In the end, the screen did not uncover new insight into the mechanism of the UPR, but promises to uncover new information about cytoplasmic RNA degradation. While the mutation found in \textit{SKI2} may not provide new information about UPR mechanism, it may prove to be a useful tool for study of 3′-5′ cytoplasmic RNA degradation. Very little is known about the exact mechanism of Ski2 function during RNA degradation and
characterization of mutations such as this could provide great insight into how Ski2 operates. It has been suggested that helicases, such as Mtr4 and Ski2, could serve to provide the exosome with a primed mRNA substrate by recognizing substrate and removing secondary structure and RNA binding proteins prior to degradation (Houseley and Tollervey, 2009). Accordingly, the structure of yeast Ski2 was recently solved (Halbach et al., 2012) and mapping of our SOR2 mutation reveals that the G1361A mutation, encoding for an arginine to histidine substitution at residue 454, is in a prime location for interaction with RNA substrate (Figure 5). The ski2(G1361A) mutant could reveal more about how the protein interacts with mRNA, Ski3, Ski8, Ski7 and the exosome. The other SOR2 mutations may be hiding other previously unidentified components of RNA degradation pathways.
Table 1. List of SOR2 Mutants

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### Table 2. Yeast Strains

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Table 3. Oligonucleotide Primers used for Cloning
Table 4. Oligonucleotide Primers used for Gene Sequencing

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Figure 1. Growth of trl1-100, Δski2::kanMX6, pRS315-ski2(G1361A) Strain

PWy712 (the original SOR2 41.1 trl1-100), PWy610 (trl1-100) and yCR95
(Δski2::kanMX6 carrying pRS315-ski2(G1361A) as the sole copy of SKI2) were plated
onto YPD (control) or YPD containing 0.25 µg/ml tunicamycin to induce the UPR. Cells
were grown to equal numbers, serially diluted 1:5 and spotted onto plates. The original
SOR2 41.1 and trl1-100 strain served as controls.
Figure 1.
Figure 2. Growth of trl1-100, ski(G1361A) Strain

Wildtype W303, PWy712 (the original SOR2 41.1 trl1-100), PWy610 (trl1-100) and yMD90 (trl1-100, ski2(G1361A)::SKI2) were plated onto YPD (control) or YPD containing 0.25 µg/ml tunicamycin to induce the UPR. Cells were grown to equal numbers, serially diluted 1:5 and spotted onto plates. W303, PWy712 and PWy610 served as control strains.
Figure 2.
Figure 3. *HAC1* mRNA Splicing in *trl1-100, ski(G1361A)* Strain

Northern blot analysis of *HAC1* splicing in wildtype W303, *PWy610 (trl1-100), PWy712* (the original *SOR2 41.1 trl1-100*) and two clones of *ski2(G1361A): yMD90* and *yMD91 (trl1-100, ski2(G1361A):::SKI2)*. UPR was induced with 8mM DTT. W303 serves as the control for wildtype *HAC1* splicing where full length *HAC1* mRNA can be seen after UPR induction. In PWy610 cells the *HAC1* mRNA is degraded after UPR induction and full length mRNA is not observed. The SOR2 strain, PWy712, stabilizes the 5’ exon and 5’exon-intron after UPR induction. The strains carrying *trl1-100* and an integrated copy of *ski2(G1361A)* show an identical splicing phenotype to that of the control SOR2 strain, PWy712.
Figure 3.

<table>
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<tr>
<th>Strain</th>
<th>W303</th>
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<th>PWy712</th>
<th>yMD90</th>
<th>yMD91</th>
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<td>+</td>
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<td>+</td>
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<td>-</td>
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<td>+</td>
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![Image of gel electrophoresis](image-url)
Figure 4. Growth of *trl1-100, ski(G1361A)* Strain carrying wildtype or splice site mutations of *HAC1*

Strains were grown to equal cell density and serial 1:5 dilutions of each strain were plated onto SC-leu or SD-leu + 0.25μg/ml tunicamycin to induce the UPR. Strain names are indicated to the left of the plates and genotypes are listed to the right.
**Figure 4.**

<table>
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SD -leu  SD -leu + 0.25μg/ml Tm
Figure 5. The ski(G1361A) Mutation Modeled onto Structures of Ski2 and Mtr4

The structures of *S. cerevisiae* Ski2 and its nuclear homolog Mtr4. a) The domain architecture of the Ski2 protein lacking the N-terminal 295 amino acids (Halbach et al., 2012). The SOR2 R454H mutation maps to the first RecA domain in the helicase region of the protein. b.) The helicase domain made up of RecA1 and RecA2 shown in teal and the helical bundle shown in lavender of Ski2 (PDB code 4a4z)(Halbach et al., 2012) modeled with the RNA as it was crystallized with Mtr4 (PDB code 2xgj)(Weir et al., 2010). The R454H mutation is shown in red. c.) The helicase domain, shown in teal, and helical bundles, shown in lavender, of Mtr4 as it was crystallized with RNA (PDB code 4a4z)(Weir et al., 2010). The analogous residue to Ski2 R454, Mtr4 R272, is shown in red. It appears that the mutation is in prime location to interact with the RNA.
Figure 5.

a.

b.

Ski2

Mtr4
References

Anderson, J.S., and Parker, R.P. (1998). The 3' to 5' degradation of yeast mRNAs is a general mechanism for mRNA turnover that requires the SKI2 DEVH box protein and 3' to 5' exonucleases of the exosome complex. EMBO J 17, 1497-1506.


Halbach, F., Rode, M., and Conti, E. (2012). The crystal structure of S. cerevisiae Ski2, a DExH helicase associated with the cytoplasmic functions of the exosome. RNA 18, 124-134.


Chapter 5

Ribosome Mapping in Budding Yeast with Aberrant Ribosomes and Translocational Machinery
Introduction

As proteins are synthesized, the majority of nascent polypeptides passively travel through the hydrophobic exit tunnel of the large ribosomal subunit. However, recent evidence suggests that some proteins are able to interact with the ribosome directly through contacts in the exit tunnel prior to emerging from the ribosome (Woolhead et al., 2006; Woolhead et al., 2004). Such interaction between the nascent polypeptide and the exit tunnel are thought to relay information to the peptidyl transferase center and modulate gene expression by controlling the rate of translation or inducing stalling of the ribosome.

This control of translation is an important means of modulating gene expression. Examples of this type of translational regulation have been readily studied in bacteria. The SecM leader peptide induces stalling of the ribosome and thus influences translation of the downstream secA gene (Nakatogawa and Ito, 2002; Woolhead et al., 2006; Yap and Bernstein, 2009). Likewise, a similar stalling mechanism controls the expression of the tnaA and tnaB genes through exit tunnel interactions with the tnaC leader peptide (Cruz-Vera and Yanofsky, 2008; Gong and Yanofsky, 2002; Seidelt et al., 2009). In both cases, mutations in the ribosome exit tunnel have been shown to alleviate pausing and impact gene expression.

In yeast cells, this type of translational regulation has been observed for genes containing regulatory upstream open reading frames (uORFs) such as those found before GCN4 and CPAI. The GCN4 mRNA encodes for a transcriptional activator of genes involved in amino acid biosynthesis. Its expression is controlled by four uORFS that repress translation of GCN4. Under conditions of amino acid starvation, two trans-acting
factors work to allow ribosomes to synthesize Gcn4 (Hinnebusch, 2005). A second example in yeast is the CPAI gene that encodes a subunit of an enzyme involved in arginine biosynthesis. It is repressed through translation of a uORF that encodes for a 25 amino acid inhibitory peptide (Hood et al., 2009).

In yeast, ribosomal protein Rpl17a (L22 in E. coli) is a prime candidate for a protein that can communicate information from the peptidyl transferase center to the exit tunnel of the ribosome. This protein is the only protein whose length spans from the peptidyl transferase center to the exit tunnel (Ban et al., 2000; Nissen et al., 2000). Structures of the large subunit of the ribosome have also revealed that the ribosomal proteins L22 and L4 in bacteria are located within the exit tunnel (Nissen et al., 2000). Together they form a constriction and create the narrowest point within the tunnel.

In our lab, Isabella Halama showed that mutations in the conserved loop of Rpl17a that protrudes into the exit tunnel have a severe growth defect. However, global translation is the same as wildtype and the mutant protein incorporated into actively translating ribosomes as shown on polysome profiles. Additionally, flotation gradients revealed that the same amount of ribosomes associated with the surface of the ER membrane. Therefore, the cause of the growth defect remains unexplained.

We used these mutations in Rpl17a as a starting point for further investigation into the mechanism behind this growth defect. We hypothesize that mutations in Rpl17a eliminate the protein’s ability to interact with nascent peptides in the exit tunnel. Thus, these strains are no longer able to utilize this important form of translational control over gene expression. Here we show our efforts to use genome wide ribosome footprinting to examine ribosome location in wildtype and rpl17a mutant cells and identify genes that
utilize ribosome pausing mediated by contacts in the exit tunnel for their proper expression.

In a similar fashion, co-translational targeting modulates translation to allow for proper targeting of a subset of proteins to the ER membrane. Binding of the SRP to signal sequences induces a translational pause that is relieved upon binding to the SRP receptor. However, in genome wide ribosome footprinting of yeast, this translational pause at ribosomes has never been observed (Ingolia et al., 2009). Additionally, all signal sequence containing genes have only been predicted bioinformatically, but have never been verified \textit{in vivo}. Here, we describe an attempt to visualize these pauses in yeast by using a deletion of one of the SRP receptor subunits in yeast. The SRP will be able to bind to a signal sequence in these cells, but will not be released. We hypothesized that this will prolong the stall long enough to identify pausing on genes containing signal sequences. This would allow for a genome wide mapping of signal sequence containing genes.

\textbf{Materials and Methods}

\textit{RPL17A Yeast Strain Construction}

All plasmids, strains and oligonucleotides used in this study can be found in Table 1-3, respectively. In W303 strains, \textit{RPL17B} was deleted with natNT2 using the Pringle method of deletion (Longtine et al., 1998). The natNT2 construct was PCR amplified from pFA6a-natNT2 (Carsten et al., 2004) using the oligonucleotide primers oMD221 and oMD222. The PCR product was transformed into \textit{S. cerevisiae} strain W303-1B to create yMD51.
To construct a *URA3* marked 2µ plasmid containing *RPL17A*, the *RPL17A* was amplified from W303 yeast genomic DNA with oMD199 and oMD200 using *Pfu* TURBO (Strategene). These oligonucleotide primers added *XmaI* and *XhoI* restriction sites flanking the *RPL17A* gene product. The amplified gene was inserted into pRS426 using the *XmaI* and *XhoI* restriction sites. This created plasmid pMD29.

Mated pMD51 (W303, MATα, Δrpl17b::natNT2) with a W303 MAT a strain to yield yMD55. The *RPL17A* gene was deleted in the yMD55 diploid strain using the Pringle method of deletion (Longtine et al., 1998). A deletion construct was created from pFA6a-kanMX6 (Longtine et al., 1998) using oMD203 and oMD204. This created a diploid strain, yMD58, containing deletions of both rpl17a and rpl17b in one copy of each gene. A wildtype copy of *RPL17A* was transformed into this strain on pMD29 (pRS426-*RPL17A*) to create yMD59. This diploid strain was sporulated using standard techniques and tetrads were dissected. To genotype the haploid strains, dissected colonies were replica plated onto plates lacking uracil or containing antibiotics to select for kan and nat resistance.

A variety of mutations were made in the *RPL17A* gene. The mutations were made in pMD29 using a single oligonucleotide mutagenesis technique (Makarova et al., 2000). The mutations made are listed in Table 5-4 using oligos oMD231-oMD240. Sequenced to confirm mutagenesis. All point mutations were subcloned into pRS425 and pRS306. The extra *XbaI* site in pRS306 was removed by PCR mutagenesis using oMD246 to allow for cloning into the *XbaI* site located in the multiple cloning region of the vector.

To create strains containing the desired rpl17a-*H133A,R135A* mutation a loop-in, loop-out technique was utilized. pMD42 (pRS306-rpl17a-*H133A,R135A*) was linearized
within the \textit{rpl17a} gene using \textit{MscI}. The linearized plasmid was transformed into yMD51 (W303, MATa, \textit{\textDelta}rpl17b::natNT2). The correct integration of this plasmid was confirmed using colony PCR with oMD220 and oMD263 and subsequent digestion with \textit{SacI} to confirm the presence of the desired mutation. The \textit{URA3} gene was then looped out by growth overnight in rich media followed by selection on 5-FOA. Colonies were screened by colony PCR and sequencing to select cells that contained only the mutant \textit{rpl17a-} \textit{H133A,R135A}. This produced the strain yMD66. A strain bearing the wildtype \textit{RPL17A} was also selected, yMD65.

To construct strains to look at GCN4 activation, PCR amplified the \textit{HIS3} gene from pRS313 using oligonucleotide primers oMD273 and oMD274. Transformed the gel purified PCR product into yMD51, yMD65 and yMD66 to repair \textit{HIS3} gene. Selected for positives on SD–his plates. This created strains identical to yMD51, yMD65 and yMD66 with repaired \textit{HIS3} genes called yMD73, yMD74 and yMD75, respectively. Linearized the pRS306-GCN4-GFP (Chubukov et al., 2012) (pMD67) reporter with \textit{PpuMI} and integrated into the \textit{URA3} gene.

\textbf{\textit{\textDelta}srp101} Yeast Strain Construction

Efforts were made to delete the \textit{SRP101} and \textit{SRP102} genes in \textit{S. cerevisiae} using the Pringle method (Longtine et al., 1998). However, we were unable to make these deletions. These strains had been made successfully in our lab previously (Ogg et al., 1992). Therefore, they were pulled from the Walter Lab collection. The diploid strain PWy84 (\textit{\textDelta}srp101/SRP101) was obtained and sporulated using standard procedures. The
resulting spores were genotyped. Both a wildtype MATa, SRP101 strain and a MATa, ∆srp101::ADE2 strain were selected and named yMD72 and yMD71, respectively.

**Plasmid Shuffle Assay**

To test for the viability of the various RPL17A mutations, a plasmid shuffle assay was performed. The yeast strain yMD57 (W303, MATα, ∆rpl17b::natNT2, ∆rpl17a::kanMX6, pRS426-RPL17A) was transformed with pRS425 vectors containing wildtype RPL17A or one of the ten mutations to be tested. Positive transformants were selected for on media lacking leucine. Grew transformants for 2-3 days at 30°C. The colonies were replica plated onto plates containing 5-Fluoroornotic Acid (5-FOA) to select against pMD29 (pRS426-RPL17A). Viability on 5-FOA plates was then determined.

**Measuring Yeast Growth Rates**

Overnight cultures of yeast strains were grown at 30°C. Timepoints were taken and OD₆₀₀ of the culture was measured. These cell densities were plotted and the doubling times of the strains were calculated.

**Ribosome Footprinting**

Ribosome footprinting was performed essentially as previously described (Ingolia et al., 2009). Yeast strains were grown overnight at 30°C and diluted into 1L of rich media. Growth was continued at 30°C until and OD₆₀₀ of approximately 0.6 was reached. At this time, cells were treated with a final concentration of 100µg/ml of cycloheximide and incubated for an additional 2 minutes at 30°C with shaking. Cells were then
harvested by filtration. A small amount of cells were removed to a cryovial for total RNA isolation and frozen in liquid nitrogen. The remainder was resuspended in polysome lysis buffer (20mM Tris pH 8.0, 140mM KCl, 1.5mM MgCl₂, 100µg/ml cycloheximide, 1% Triton X-100) and frozen dropwise into liquid nitrogen. Cells were lysed by pulverization in a Retsch Mixer Mill for a total of 6x 3 minute intervals at 15Hz under liquid nitrogen. Extract was thawed at 30˚C for 5 minutes, clarified at 3,000xg for 5 minutes at 4˚C and clarified again at 20,000xg for 10 minutes at 4˚C. The OD₂₆₀ of the cleared extract was determined on a Nanodrop.

For nuclease digestion, 50 OD₂₆₀ units of extract were incubated with 750 units of RNase I (Invitrogen) at 25˚C for 1 hour. The reaction was quenched with 400 units of SUPERase-In (Ambion). A control reaction lacking nuclease was performed in parallel with 50 OD₂₆₀ units of extract. Monosome fractions were separated over a 10-50% sucrose gradient centrifuged for 3 hours at 35,000 rpm, 4˚C in an SW41 rotor and isolated with a BIO-COMP fractionator. RNA was isolated using a standard hot phenol-chloroform extraction followed by isolation over a YM-100 microconcentrator (Amicon).

Total RNA was isolated from frozen cells using a standard hot phenol-chloroform extraction. The mRNA fraction was then isolated from 150µg of total RNA in 60µl total volume. An equal volume of 2x binding buffer (20mM Tris pH 7.0, 1M LiCl, 6.7mM EDTA) was added to RNA. Samples were denatured at 80˚C for 2 minutes. RNA was incubated with 150µl of oligo-dT dynabeads (Invitrogen) that had been washed and supernatant removed. mRNA was bound to beads for 5 minutes at room temperature, washed twice with wash buffer B (10mM Tris pH 7.0, 0.15M LiCl, 1mM EDTA). The mRNA was then eluted in 40µl 10mM Tris pH 7.0 at 80˚C for 2 minutes. Isolated mRNA
was fragmented in an equal volume of 2x fragmentation buffer (2µl 0.5M EDTA, 60µl 0.1M Na₂CO₃, 440µl 0.1M NaHCO₃) at 95°C for 30 minutes.

Fragmented mRNA and monosome footprints were then gel purified on 15% TBE-Urea polyacrylamide gels (Invitrogen). RNA was recovered from the gel and the 3’ ends were dephosphorylated using T4 polynucleotide kinase (NEB) at 37°C for 1 hour. A DNA linker (1ug miRNA Linker 1 from IDT) was ligated to the dephosphorylated 3’ end of all samples using T4 RNA ligase 2 (NEB). The linker ligated RNA was then gel purified over 10% TBE-Urea polyacrylamide gels and RNA fragments were recovered.

rRNA contaminants were subtracted from the monosome samples only as previously described (Brar et al., 2011).

Both mRNA and footprint samples were reverse transcribed using Superscript III (Invitrogen) and gel purified over 10% TBE-Urea polyacrylamide gels. The single stranded DNA was then circularized using CircLigase (EPICENTRE). The circular DNA was used as a PCR template in a short non-saturating PCR reaction to generate linear double stranded DNA fragments. The PCR product was then examined on non-denaturing 8% TB polyacrylamide gels and DNA fragments were purified from the gel. The double stranded DNA was quantitated on a Bioanalyzer using a high sensitivity DNA chip.

Cluster generation and sequencing was performed on the Solexa in the Center for Advanced Technology (CAT) at UCSF following the manufacturers protocol. The sequencing primer used was 5’-CGACAGGTTTCAGTTCTACAGTCCGACGATC-3’).
**Data Analysis**

Rudimentary data analysis was performed using programs written by N. Ingolia as was previously described (Ingolia et al., 2009).

**Western Blotting**

Grew overnight liquid cultures in 2x SC-his media at 30°C. Harvested 1.5 OD$_{600}$ units of each of the cells and froze pellets in liquid nitrogen for 0 timepoint. Induced amino acid starvation with 50mM final concentration of 3-aminotriazole (3-AT). Harvested timepoints as above at 10, 20 and 40 minutes after 3-AT addition. TCA precipitated protein as follows. Resuspended pellet in 150µl of Solution 1 (1.85M NaOH, 7.4% BME) and incubated on ice for 10 minutes. Added 150µl 50% TCA and incubated on ice for 10 minutes. Centrifuged for 2 minutes at maximum speed at 4°C. Removed supernatant and washed pellet in 1ml acetone. Resuspended pellets in 100µl of 1x protein loading buffer. Boiled protein for 5 minutes and loaded 10µl on 10%, 10 well, 1.0mm bis-tris gel (Invitrogen). Ran gel in 1x MOPS buffer at 180V for 55 minutes and transferred protein to 0.2µM nitrocellulose membrane. Blocked membrane in 5% milk/PBST overnight at 4°C. Incubated membrane in anti-phospho-eIF2α antibody (Cell Signaling, 9721) diluted 1:1000 in 5% milk/PBST for 1 hour at room temperature. Washed in PBST. Incubated in anti-mouse secondary antibody (Pierce) diluted 1:50,000 in 5% milk/PBST for 1 hour at room temperature. Developed with Dura ECL (Pierce) and exposed to film.
Flow Cytometry

Flow cytometry was performed to measure GCN4. Grew cells overnight in 2x SC-his media. Induced amino acid starvation with the addition of 50mM final concentration of 3-AT. Flow cytometry performed as described (Pincus et al., 2010).

Results

Mutant rpl17a Ribosome Footprinting

We constructed new mutations in the RPL17A gene rather than use strains in the collection made by Isabella Halama in order to incorporate more recent structural and biochemical work. Mutations were made in RPL17A based on homology to the E. coli L22 protein and using a recent cryo-electron microscopy structure of the E. coli 70S ribosome stalled during the translation of the TnaC leader peptide by the addition of (Seidelt et al., 2009). This structure revealed 23S rRNA nucleotides and residues from loops of L22 and L4 that made direct contact with the TnaC leader peptide tryptophan (Figure 1)(Seidelt et al., 2009). The residues that were chosen for mutation are shown in Table 4. We chose a combination of point mutations in the loop of Rpl17a that protrudes into the exit tunnel and complete deletions of this loop.

We began by testing the mutations for viability in a plasmid shuffle assay. As most ribosomal protein genes have two copies in yeast that can readily substitute for one another, typically called a and b, we constructed a strain with the genes encoding Rpl17a and Rpl17b deleted. Double deletion of both these genes is not viable in yeast; therefore a wildtype RPL17A covered the deletions on a high copy yeast URA3 marked 2µ plasmid. As yeast cells prefer to have two copies of the ribosomal protein genes, when one copy of
the ribosomal protein is deleted, cells can become aneuploid for the chromosome containing the remaining copy of the gene. Additionally, many ribosomal proteins are sensitive to copy number. Therefore, we reasoned that this gene would best be covered by the high expression provided by a 2µ plasmid. Doubling time for these strains was slightly increased when compared to wildtype, indicating that $RPL17A$ on the plasmid was not completely complementing a wildtype situation (Figure 2). We then transformed $LEU2$ marked 2µ plasmids containing each of the ten mutations that were tested, shuffled out the $URA3$ marked wildtype copy and scored for viability. Only the point mutations were able to substitute for the wildtype copy of $RPL17A$ (Table 4).

In order to choose which mutations to characterize, we determined the doubling time for all of the viable $rpl17a$ mutations using a growth assay in liquid culture. In this assay, the mutated genes were all expressed on $LEU2$ marked 2µ plasmids. Surprisingly, the single point mutations H133A, R135A, and G134S all grew at same rate as the wildtype $RPL17A$ control strain (Figure 3). This is contrary to the observations made by Isabella Halama that were the impetus for this project. In her assay, the H133A mutation had a severe growth defect and only the R135A mutation grew with wildtype kinetics. As we set out to determine why cells with mutations in their ribosome exit tunnel had growth defects, the ideal strain would show a severe growth defect when compared to wildtype. The double H133A, R135A mutation showed a marked difference in doubling time (Figure 3), therefore we chose to move forward with this mutation.

The double point mutation of H133A, R135A was then integrated into the $RPL17A$ locus in a $Δrpl17b$ strain background. The doubling time of this strain was measured using a growth assay in liquid media. While the growth defect was not as
severe as that seen when the mutant protein was expressed on a 2μ plasmid, the doubling time was still 40 minutes longer than the control strain, a wildtype RPL17A in a Δrpl17b strain background (Figure 4).

Polysome profile analysis to look at the general status of translation in these cells was performed together with monosome isolation for ribosome footprinting. While the rpl17a strain was active in translation, we observed a distinct increase in free 60S large ribosomal subunits (Figure 6) when compared to that of the wildtype RPL17A strain (Figure 5). This may indicate that the mutant strains have a ribosome assembly defect or a slowed rate of initiation. This is contrary to what was observed by Isabella Halama. Her strains showed a marked increase in free 40S small ribosomal subunits.

Ribosome footprinting was performed from isolated monosome fractions (Figures 5 and 6). Footprinting was successful and we obtained over 8 million reads mapping to yeast genes (Table 5). Generally, we did not observe much difference in ribosome density on individual genes between wildtype and mutant ribosomes (Figure 7 to 10). However, there were a few outliers that we did not follow up on.

In general, data was analyzed qualitatively by looking gene to gene using the Mochiview genome browser (Homann and Johnson, 2010). Visualizing the ribosome locations in this manner allowed us to identify two interesting elements in our data. The first standout feature in our data was the misregulation of GCN4 (Figure 11). GCN4 has been extensively studied as a translationally regulated gene in yeast. Its expression is controlled by four uORFs in its 5’ UTR and is induced upon amino acid starvation (Hinnebusch, 2005). We observed a complete loss of ribosome density at the uORFs in our rpl17a mutation, yet this was not accompanied by the expected increase in GCN4
translation (Figure 11). We also noted an increase in ribosome occupancy in the region upstream of the known uORFs as was previously described during conditions of starvation (Ingolia et al., 2009).

This dramatic misregulation of GCN4 led us to question if the rpl17a mutant was trying to respond to stress or if the mutant strain was capable of responding to amino acid starvation. A Western blot against phosphorylated eIF2α showed that the rpl17a does not phosphorylate eIF2α in the absence of amino acid stress (Figure 12). Furthermore, when stress in the form of 3-AT is applied to the cells, the mutant strains are capable of phosphorylating eIF2α to the same degree as wildtype (Figure 12). Additionally, the mutant rpl17a cells can activate a reporter of GCN4 under amino acid starvation as well as their wildtype counterparts (Figure 13).

The second standout feature in our data was the loss of ribosome density over the start codon of genes (Figure 14). This dramatic difference was easily observed qualitatively in the genome browser, as shown for the genes CPR5 and OST6 (Figure 15). Additionally, when the fraction of reads are plotted for every gene as a function of the distance from the start codon, it becomes apparent that this is a global feature in our mutant (Figure 14). GO analysis was performed on genes where the mutant has less than 5% of ribosome occupancy over the AUG as the wildtype strain, however this was not followed up on (Figure 16). This loss of ribosome density could indicate that the rpl17a mutation causes an initiation defect.
**Δsrp101 Ribosome Footprinting**

It has been established that budding yeast can survive without the SRP targeting machinery (Hann and Walter, 1991). In yeast, the SRP receptor is a heterodimeric ER membrane protein composed of SRα and SRβ, encoded by *SRP101* and *SRP102*, respectively. Deletions of both *SRP101* and *SRP102* are viable in yeast, although the strains grow with significantly reduced kinetics (Ogg et al., 1998; Ogg et al., 1992). Here, efforts to construct these strains were unsuccessful. Every attempt yielded a wildtype gene that was able to grow on selective media. Instead, diploid strains carrying Δ*srp101::ADE2/SRP101* were obtained from the Walter lab collection (Ogg et al., 1992). These strains were sporulated successfully. A strain carrying Δ*srp101::ADE2* was obtained along with a control strain with wildtype *SRP101*. The doubling times of these strains were determined (Figure 17). Polysome analysis of these strains showed that active translation was nearly identical to that of the wildtype strain (Figures 18 and 19).

We successfully performed the ribosome footprinting protocol using these strains and obtained over 6 million reads per sample (Table 6). Using general data analysis across entire genes we were unable to see any significant differences between the samples (Figures 20 to 23).

**Discussion**

**Mutant rpl17a Ribosome Footprinting**

Genome-wide ribosome footprinting, which reveals the precise location of ribosomes at any given time, is a powerful technique for understanding translational regulation. The study of the *rpl17a* mutation could shed light on how the eukaryotic
ribosome utilizes contacts in the exit tunnel to modulate translation. Our hypothesis was that eukaryotic ribosomes, like their prokaryotic counterparts, use the constriction made by RPL17A to regulate translation by modulating translation rates and inducing pausing through direct contacts with specific polypeptides as they are synthesized. Mutations in the loop that forms this constriction should alleviate this pausing and allow us to identify specific genes, mRNA sequences and mRNA structures that are subject to regulation in yeast.

There are many perplexing observations that were made during the course of these experiments that we have yet to explain. First, we observed an increase in the 60S large ribosomal subunits in the rpl17a mutant cells, yet these strains appear to have the same number of polysomes as the wildtype strain (figure 5-). Similar experiments with E. coli L22 mutant ribosomes show no polysomes (Oh, E and Weissman, J, personal communication). Secondly, we do not yet have an explanation for the aberrant regulation of the GCN4 gene. The cells do not appear to be sensing starvation stress when none is present, as they do not phosphorylate eIF2α (figure 5-). Additionally, when the mutant cells encounter amino acid starvation stress they are able to respond and activate GCN4 (figure 5-). Lastly, the loss of ribosome density at start codons appears to indicate that the mutant cells have a translational initiation defect. This is an interesting observation that we do not yet understand.

While this data looks promising, recent developments in the protocol for ribosome footprinting lead us to strongly suggest that the ribosome footprinting of these strains be repeated. We used cycloheximide to lock ribosomes into place prior to nuclease digestion and monosome isolation. However, it is now thought that cycloheximide may be causing
artifacts in the footprinting data. Cycloheximide may allow another round of translation to occur before ribosomes are actually stalled in place. This is not necessarily a problem when looking at the total ribosome density on a gene. However, the interpretation of this data relies heavily on precise positional data. With this in mind, the interesting observations that we made could simply be an artifact of cycloheximide use. The new protocol for ribosome footprinting uses flash freezing of the cells directly into liquid nitrogen and eliminates the use of cycloheximide. It would be very interesting to repeat the genome wide ribosome footprinting on the rpl17a strain using the newest techniques available.

A large obstacle for analyzing this data set was bioinformatics. This data could benefit from the expertise of someone who can readily program and analyze this data in a more rigorous fashion. Ideally, we would like to use this data to identify mRNA sequences where ribosome pausing is lost in the rpl17a mutant. To achieve this, a program needs to be developed to perform a systematic analysis by position and calculate a ribosome stall score by position at the codon level.

**Δsrp101 Ribosome Footprinting**

The co-translational targeting mechanism of the SRP is highly conserved. It is essential in most every organism studied to date, except for budding yeast. We still do not know why budding yeast can live without the SRP, but they must rely heavily on post-translational translocation methods. In eukaryotic cells, the SRP induces a translational pause after signal sequence binding that is released upon interaction with the SRP receptor and subsequent transfer to the translocon. However, such pauses were not
observed when genome wide ribosome mapping was performed on yeast (Ingolia et al., 2009). In order to study how SRP influences ribosome occupancy in yeast and hopefully capture these transient pauses, we utilized the unique ability that yeast has to live without the SRP. We hypothesized that by deleting one copy of the SRP receptor we could freeze the SRP at signal sequences, as the pause could not be relieved by the SRP receptor. Unfortunately, we failed to see any ribosome pausing occurring at signal sequences even in strains lacking the SRP receptor. As stated above, after these experiments were performed, it was discovered that the addition of cycloheximide to lock ribosomes in place onto mRNA was producing artifacts in the data. It is believed that the cycloheximide is allowing another round of elongation to occur on the mRNA prior to stalling. As we are looking for very positional specific defects, rather than total ribosome occupancy on a gene, this creates some serious problems for interpretation of this data. It is highly likely that the use of cycloheximide is obscuring our ability to capture ribosome pausing at signal sequences. Currently a technique where cells are rapidly frozen in liquid nitrogen, rather than treated with cycloheximide, is being utilized to “freeze” ribosomes in place. Therefore, this data should be recollected using the newest ribosome footprinting and sequencing techniques.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>yMD51</td>
<td>W303; MAT α; ade2-1; trp1-1; can1-100; leu2-3,112; his3-11,15; ura3-1; psi+; Δrpl17b::natNT2</td>
</tr>
<tr>
<td>yMD55</td>
<td>W303; MAT a/MAT α; ade2-1/ade2-1; trp1-1/trp1-1; can1-100/can1-100; leu2-3,112/leu2-3,112; his3-11,15/his3-11,15; ura3-1/ura3-1; RPL17B/Δrpl17b::natNT2</td>
</tr>
<tr>
<td>yMD57</td>
<td>W303; MAT α; ade2-1; trp1-1; can1-100; leu2-3,112; his3-11,15; ura3-1; psi+; Δrpl17b::natNT2; Δrpl17a::kanMX6, pRS426-RPL17A</td>
</tr>
<tr>
<td>yMD58</td>
<td>W303; MAT a/MAT α; ade2-1/ade2-1; trp1-1/trp1-1; can1-100/can1-100; leu2-3,112/leu2-3,112; his3-11,15/his3-11,15; ura3-1/ura3-1; RPL17B/Δrpl17b::natNT2; RPL17A/Δrpl17a::kanMX6</td>
</tr>
<tr>
<td>yMD59</td>
<td>W303; MAT a/MAT α; ade2-1/ade2-1; trp1-1/trp1-1; can1-100/can1-100; leu2-3,112/leu2-3,112; his3-11,15/his3-11,15; ura3-1/ura3-1; RPL17B/Δrpl17b::natNT2; pRS426-RPL17A</td>
</tr>
<tr>
<td>yMD65</td>
<td>W303; MAT α; ade2-1; trp1-1; can1-100; leu2-3,112; his3-11,15; ura3-1; psi+; Δrpl17b::natNT2</td>
</tr>
<tr>
<td>yMD66</td>
<td>W303; MAT α; ade2-1; trp1-1; can1-100; leu2-3,112; his3-11,15; ura3-1; psi+; Δrpl17b::natNT2; rpl17a-H133A,R135A::RPL17A</td>
</tr>
<tr>
<td>yMD71</td>
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</tr>
<tr>
<td>yMD72</td>
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</tr>
<tr>
<td>yMD73</td>
<td>W303; MAT α; ade2-1; trp1-1; can1-100; leu2-3,112; HIS3; ura3-1; psi+; Δrpl17b::natNT2</td>
</tr>
<tr>
<td>yMD74</td>
<td>W303; MAT α; ade2-1; trp1-1; can1-100; leu2-3,112; HIS3; ura3-1; psi+; Δrpl17b::natNT2; rpl17a-H133A,R135A::RPL17A</td>
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<tr>
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### Table 2. Plasmids

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene Involved</th>
<th>Vector Backbone</th>
<th>Marker</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMD29</td>
<td>RPL17A (670 upstream, 335 downstream)</td>
<td>pRS426</td>
<td>URA3</td>
<td>this study</td>
</tr>
<tr>
<td>pMD42</td>
<td>rpl17a-H133A,R135A</td>
<td>pRS306</td>
<td>URA3</td>
<td>this study</td>
</tr>
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<td>pMD67</td>
<td>GCN4-GFP reporter</td>
<td>pRS306</td>
<td>URA3</td>
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<tr>
<td>Name</td>
<td>5'-3' Sequence</td>
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<td>---------</td>
<td>----------------</td>
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</tr>
<tr>
<td>oMD203</td>
<td>gccaacaaagagtcctcaagaactactaataagattaaacGGATCCCGGTAAATTAA</td>
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<td></td>
<td></td>
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<tr>
<td>oMD204</td>
<td>cagagaattatattatattatatattttctcttcttagATCGTGCAGCTCG</td>
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<tr>
<td>oMD221</td>
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<tr>
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<tr>
<td>oMD231</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oMD232</td>
<td>gaagaactttacagctcagctGCaatcaacaagttagcagtttctcc</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>oMD233</td>
<td>gaagaactttacagctcagAtgaatacaacaagttagcagtttctcc</td>
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<td></td>
<td></td>
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<tr>
<td>oMD234</td>
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<td>oMD235</td>
<td>gaagaagaactttacagctGCggtGCaatcaacaagttagcagtttctcc</td>
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<tr>
<td>oMD236</td>
<td>caaagcaagaagaagaactttaCAatcaacaagttagcagtttctccatcc</td>
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<tr>
<td>oMD237</td>
<td>ccaagaagaagaactttacagAAgaatcaacaagttagcagtttctcc</td>
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<td>oMD238</td>
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<td>oMD239</td>
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<td>oMD240</td>
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<tr>
<td>oMD263</td>
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<td>oMD273</td>
<td>caagataacaggcaag</td>
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<tr>
<td>oMD274</td>
<td>ctgcagctttttaatcgg</td>
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Table 4. Summary and Viability of Rpl17a Mutations

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Viability</th>
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</thead>
<tbody>
<tr>
<td>H133A</td>
<td>++</td>
</tr>
<tr>
<td>R135A</td>
<td>++</td>
</tr>
<tr>
<td>G134D</td>
<td>++</td>
</tr>
<tr>
<td>G134S</td>
<td>-/+</td>
</tr>
<tr>
<td>H133A, R135A</td>
<td>+</td>
</tr>
<tr>
<td>Δ 132, 133, 134</td>
<td>-</td>
</tr>
<tr>
<td>Δ 131, 132, 133, 134, 135</td>
<td>-</td>
</tr>
<tr>
<td>Δ 133, 134, 135</td>
<td>-</td>
</tr>
<tr>
<td>R131A, H133A, G134A, R135A</td>
<td>-/+</td>
</tr>
<tr>
<td>Δ 132, 133, 134, 135, 136</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 5. Number of reads from rpl17a Ribosome Footprinting

<table>
<thead>
<tr>
<th></th>
<th>RPL17A</th>
<th>rpl17a-H133A,R135A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Reads</td>
<td>19337641</td>
<td>20518060</td>
</tr>
<tr>
<td>rRNA Fragments</td>
<td>10162405</td>
<td>10379944</td>
</tr>
<tr>
<td>Non-rRNA</td>
<td>9175236</td>
<td>10138116</td>
</tr>
<tr>
<td>Aligned to chromosome</td>
<td>8824038</td>
<td>9220028</td>
</tr>
<tr>
<td>Unaligned</td>
<td>61784</td>
<td>918088</td>
</tr>
</tbody>
</table>
Table 6. Number of reads from \( \Delta \text{srp101} \) Ribosome Footprinting

<table>
<thead>
<tr>
<th></th>
<th>SRP101</th>
<th>( \Delta \text{srp101} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Reads</td>
<td>19817499</td>
<td>18051098</td>
</tr>
<tr>
<td>rRNA Fragments</td>
<td>8257308</td>
<td>10466043</td>
</tr>
<tr>
<td>Non-rRNA</td>
<td>11560191</td>
<td>7585055</td>
</tr>
<tr>
<td>Aligned to chromosome</td>
<td>10840141</td>
<td>6954945</td>
</tr>
<tr>
<td>Unaligned</td>
<td>720050</td>
<td>630110</td>
</tr>
</tbody>
</table>
Figure 1. Schematic Illustrating Contacts Made between the Ribosome and the TnaC Leader Peptide

Cartoon showing the contacts made by *E. coli* ribosomal proteins L22, the homolog of Rpl17a, and L4, the homolog of Rpl4a, with the TnaC leader peptide (adapted from Seidelt et al., 2009). Together, these two proteins form a constriction within the exit tunnel. The structure that this cartoon was based on was used to inform the mutations made in Rpl17a (Seidelt et al., 2009). Key residues where mutations were made in the Rpl17a protein are indicated with red asterisks. Residue K90 of L22 is equivalent to H133 of Rpl17a. Residue R92 of L22 is equivalent to R135 of Rpl17a.
Figure 2. Growth Rates of Δrpl17b, 2µ RPL17A Strains

Growth curves showing the doubling times of strains expressing RPL17A on a high copy 2µ plasmid. Cells were grown in liquid culture at 30°C. Timepoints were taken and the OD$_{600}$ was determined as a measure of cell density.
Figure 2.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Doubling Time</th>
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</thead>
<tbody>
<tr>
<td>W303</td>
<td>94</td>
</tr>
<tr>
<td>Δrpl17b</td>
<td>103</td>
</tr>
<tr>
<td>2u RPL17A (Ura)</td>
<td>130</td>
</tr>
<tr>
<td>2u RPL17A (Leu)</td>
<td>130</td>
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</table>
Figure 3. Growth Rates of *rpl17a* Point Mutant Strains

Growth curves showing the doubling times of strains expressing *rpl17a* mutant genes on a high copy 2μ plasmid in the Δ*rpl17b* strain background. Cells were grown in liquid culture at 30°C. Timepoints were taken and the OD₆₀₀ was determined as a measure of cell density.
Figure 3.

**Growth Rates of rpl17a mutants**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Doubling Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPL17A (WT)</td>
<td>143</td>
</tr>
<tr>
<td>H133A</td>
<td>143</td>
</tr>
<tr>
<td>R135A</td>
<td>143</td>
</tr>
<tr>
<td>G134S</td>
<td>158</td>
</tr>
<tr>
<td>H133A, R135A</td>
<td>273</td>
</tr>
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</table>
Figure 4. Growth Rate of Integrated rpl17a-H133a, R135A Mutant Strain

Growth curves showing the doubling times of strains expressing an integrated copy of rpl17a-H133A,R135A at the RPL17A locus in the Δrpl17b::natNT2 strain background. Cells were grown in liquid culture at 30°C. Timepoints were taken and the OD$_{600}$ was determined as a measure of cell density.
Figure 4.

*Growth Curve of Integrated rpl17a-35*

<table>
<thead>
<tr>
<th>Strains</th>
<th>Doubling Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Δrpl17b</td>
<td>110</td>
</tr>
<tr>
<td>Δrpl17b, rpl17a-35</td>
<td>150</td>
</tr>
</tbody>
</table>
Figure 5. Polysome and Monosome Profiles of Rpl17a Strains

Polysome profile analysis of wildtype Rpl17a strains shown in blue. Profile of monosomes after nuclease digestion and prior to isolation for ribosome footprinting shown in red. Experiment was performed in duplicate.
Figure 5.

RPL17A polysomes - replicate 1

RPL17A polysomes - replicate 2
Figure 6. Polysome and Monosome Profiles of rpl17a-H133A,R135A Strains

Polysome profile analysis of rpl17a-H133,R135 strains shown in blue. Profile of monosomes after nuclease digestion and prior to isolation for ribosome footprinting shown in red. Experiment was performed in duplicate.
Figure 6.
Figure 7. Histogram of Ribosome Footprint Density Comparing Wildtype and Mutant Rpl17a Strains
Figure 8. Ribosome Footprint Densities in Rpl17a Strains
### Less Ribosome Density in Mutant

**Category**
- aromatic compound metabolic process [GO:0006725]
- amino acid biosynthetic process [GO:0008652]
- arginine biosynthetic process [GO:0006526]
- organic acid metabolic process [GO:0006082]
- ornithine biosynthetic process [GO:0006592]
- nitrogen compound metabolic process [GO:0006807]
- oxidation reduction [GO:0055114]
- agglutination during conjugation with cellular fusion [GO:0000752]
- amino acid and derivative metabolic process [GO:0006519]
- cell adhesion [GO:0007155]
- sulfate assimilation [GO:0000103]
- methionine biosynthetic process [GO:0009086]
- metabolic process [GO:0008152]
- glyoxylate cycle [GO:0006097]
- methionine metabolic process [GO:0006555]
- cysteine biosynthetic process [GO:0019344]
- hexose transport [GO:0008645]
- arginine biosynthetic process via ornithine [GO:0042450]
- ornithine metabolic process [GO:0006591]
- citrulline metabolic process [GO:0000052]
- argininosuccinate metabolic process [GO:0000053]
- neutral amino acid transport [GO:0015804]
- guanine nucleotide transport [GO:0001408]

### More Ribosome Density in Mutant

**Category**
- rRNA metabolic process [GO:0016072]
- cell wall organization and biogenesis [GO:0007047]
- drug transport [GO:0015893]
- response to acid [GO:0001101]
- RNA modification [GO:0009451]
- thiamin and derivative metabolic process [GO:0042723]
- high-affinity zinc ion transport [GO:0006830]
- chaperone cofactor-dependent protein folding [GO:0051085]
- glyoxylate catabolic process [GO:0009436]
- riboflavin transport [GO:0003218]
- ribosome biogenesis [GO:0042254]
Figure 10. Analysis of Fragment Lengths of Ribosome Protected Fragments in Rpl17a Strains
Figure 11. Ribosome Density Across $GCN4$

Ribosome footprint densities on the $GCN4$ gene in wildtype Rpl17a, shown in pink, and mutant rpl17a, shown in teal, profiles. Gene is on the minus strand therefore 5’ end of gene is on the right of the diagram. Plot should be read right to left. This shows the loss of ribosome density at the 5’ uORFs in the rpl17a mutant strain.
Figure 12. eIFα Phosphorylation in Rpl17a Mutant Strains

Western blot for phospho-eIFα in Rpl17a strains at 0, 10, 20 and 40 minutes after amino acid starvation induced by 3-AT addition. The rpl17a mutant strains do not sense amino acid starvation stress prior to incubation with 3-AT and can mount a response similar to wildtype.
Figure 12.

![Graph showing expression levels of RPL17A and rpl17a proteins after 50mM 3AT treatment over time. The graph includes markers at 0, 10, 20, and 40 time points, with protein bands at 70, 55, 35, and 27 positions.]
Figure 13. Rpl17a Mutant Strains Respond to Amino Acid Starvation at Wildtype Levels

Use of a reporter of GCN4 promoter activity to measure the response of mutant rpl17a-H133A,R135A to stress. a) Shows the schematic of the reporter with the 5’UTR of GCN4 driving the expression of GFP. b) The response of rpl17a-H133A,R135A strains to amino acid starvation stress induced by 3-AT as measured by GFP expression using flow cytometry. Kinetics of the response are similar to wildtype.
Figure 13.

a.  

b. GCN4-GFP Reporter in Response to Starvation

Time after 50mM 3AT (minutes)

Fold Change

RPL17A

rpl17a
Figure 14. Read Density as a Function of Position in Rpl17a Strains
Figure 15. Ribosome Density Across Sample Genes with Decreased Ribosome Occupancy at the Start Codon

Ribosome density on sample genes that illustrate the loss of ribosomes at the AUG that was observed in rpl17a mutant cells. Footprints corresponding to wildtype strains are shown in pink and footprints from mutant rpl17a strains are in teal. CPR5 is on the minus strand of DNA and 5’ of the gene is on the right in the diagram. Plot should be read right to left. OST6 gene is on the plus strand of DNA with the 5’ of the gene on the left in the diagram. Plot should be read left to right.
Figure 15.
Figure 16. GO Analysis of Genes with Decreased Ribosome Density near the Start Codon in rpl17a-H133A, R135A Mutant

<table>
<thead>
<tr>
<th>Category</th>
<th>p-value</th>
<th>In Category from Cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>regulation of translation</td>
<td>4.16e-06</td>
<td>CY3 ERV46 JUP50 ADE1 HEK2 COQ1 HHT1 AMN1 SHM1 RSO6 SIT4 LYS21 LYS20 SS81 MRH1 KEH1 NPL3 TOM1 URA3 MCM3 HYP2 TRP2 PAB1 GCP2 CKG1 SGF73 CLC1 CLG1 CLG2 GUS1 HXK2 YGR054W TIF4631 CRM1 DIE2 SLH1 YHL017W NCP1 SOL3 GND1 MSR3 LC33 YKL077W YMA80 PGM1 EAP1 MCM5 NASA1 CSR1 YAC14 GST6 PRP39 CLU1 ADH3 YMR190W ERG12 DOM34 PUB1 RH2 RPH2 RAP1 TIM23 ITR2 SER1 MCA1 YOR302W GDS1 KAP120 POC4 FAS2 GLN1 SUE7 YPR007W ASN1</td>
</tr>
<tr>
<td>protein import into nucleus, docking</td>
<td>0.000018</td>
<td>NUP60 SXM1 CSE1 KAP114 CRM1 NUP53 KAP120</td>
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<tr>
<td>biopolymer biosynthetic process</td>
<td>0.000289</td>
<td>CY3 ERV46 PAM1 LYS20 SSB1 MRH1 NPL3 CUP5 ERG4 GCP1 HXK2 ERG25 YHB1 TNA1 SOL3 GND1 CWP2 PGM1 ASH1 ECM7 HXT2 FET3 ADH3 HSC52 YNL190W RGA1 PDR5 FAS2 DPM1</td>
</tr>
<tr>
<td>metabolic compound salvage</td>
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<td>POP5 ADE1 PTC3 RFC5 LYS21 AR03 YDR352W TRP2 SEL1 ERG7 Iki1 PHS1 MRS3 YNL193W HOC1 MTD1 GST1 OSTM1 YMR130W ERG12 ERG9 RHO2 SRF1 YNL217W TIM23 YCL057W MCA1 FAS2 GLN1 YMC1 DFB1</td>
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</tbody>
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Figure 17. Growth Rates of Δsrp101 and Δsrp102 Strains

Growth curves showing the doubling times of various Δsrp101 and Δsrp102 strains. Cells were grown in liquid culture at 30°C. Timepoints were taken and the OD_{600} was determined as a measure of cell density.
Figure 17.

**Growth of SRα and SRβ Deletions**

<table>
<thead>
<tr>
<th>Strains</th>
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</table>
Figure 18. Polysome and Monosome Profiles of Srp101 Strains

Polysome profile analysis of wildtype Srp101 strains shown in blue. Profile of monosomes after nuclease digestion and prior to isolation for ribosome footprinting shown in red. Experiment was performed in duplicate.
Figure 18.
Figure 19. Polysome and Monosome Profiles of ∆srp101 Strains

Polysome profile analysis of mutant ∆srp101 strains shown in blue. Profile of monosomes after nuclease digestion and prior to isolation for ribosome footprinting shown in red. Experiment was performed in duplicate.
Figure 19.

\[
\Delta srp101 \text{ polysomes - replicate 1}
\]

\[
\Delta srp101 \text{ polysomes - replicate 2}
\]
Figure 20. Histogram of Ribosome Footprint Density Comparing Wildtype and Mutant Srp101 Strains
Figure 21. Ribosome Footprint Densities in Srp101 Strains
Figure 22. Analysis of Fragment Lengths of Ribosome Protected Fragments in Srp101 Strains
Figure 23. Read Density as a Function of Position in Srp101 Strains
References


Chapter 6

Conclusions and Perspectives
Since the discovery of the Unfolded Protein Response Element (UPRE) 20 years ago (Mori et al., 1992), our understanding of the Unfolded Protein Response has developed from a single pathway controlling a transcriptional response to a branched pathway combining transcriptional upregulation with mechanisms to decrease the protein folding load of the cell. This thesis, and the resulting paper that will be published in the first issue of eLife, initiate an exciting exploration of the UPR in fission yeast. Study of the UPR in fission yeast has proven to be exciting. It has changed our understanding of the evolution of the UPR, and has also uncovered unprecedented examples of RNA regulation with discovery of the unique truncation and translation of the Bip1 mRNA. We have only just scratched the surface exploring the UPR in fission yeast, yet it has already changed our understanding of the UPR in other organisms and raised new questions about mRNA regulation and stability.

The UPR in Fission Yeast

Our experiments in fission yeast raise exciting new questions about the regulation of Ire1’s RNase specificity. All of our data suggest that Ire1 in fission yeast cleaves the Bip1 mRNA amongst other ER targeted mRNAs. However, in vitro cleavage assays would prove that Ire1 rather than another RNase is directly cleaving the Bip1 mRNA. To this end, purifying the fission yeast Ire1 protein is a critical next step. This purified protein can first be used in in vitro assays to show that Ire1 is the enzyme responsible for the observed cleavage of Bip1 and the other downregulated RNAs. In further studies, it can then be used to define a consensus sequence for fission yeast Ire1 as has already been done in budding yeast (Gonzalez et al., 1999).

We have already observed that the cleavage sites in budding yeast and fission yeast are slightly different. In vitro and in vivo assays can also probe this difference and analyze the interchangeability of these enzymes. How has this enzyme evolve from having a single substrate in budding yeast to recognizing and cleaving a wide array of mRNAs? How does fission yeast Ire1 identify which RNAs to cleave? Structural studies of fission yeast Ire1 may highlight changes in the enzyme that allow it to recognize and cleave a single mRNA substrate in budding yeast a multitude of mRNAs in fission yeast and both in metazoan cells.
In the process of studying the UPR in fission yeast, we observed the unusual stabilization of the *Bip1* mRNA despite the removal of a poly-A tail. Dissecting the mechanism of the specific stabilization of the *Bip1* mRNA will not only uncover a novel UPR signaling mechanism, but may also contribute to our understanding of the regulation of mRNAs in general. Thus far, we have little indication of how the truncated *Bip1* mRNA avoids the cell’s RNA surveillance pathways. The stabilization could mediated by an RNA binding protein, a super-stable structural element, or a modification of the 3’ end of the RNA. Our identification of this fragment via high-throughput sequencing indicates that the stabilization of the RNA does not interfere with linker ligation by T4 RNA ligase or sequencing. The fragment that aligns to the cleaved end of the *Bip1* mRNA is also highly stable compared to other mRNA fragments throughout the entire sequencing protocol. Since this protocol strips protein from mRNA, it seems unlikely that the stability is mediated by an RNA binding protein. Our favorite hypothesis is that this stabilization is induced by a covalent modification of the RNA end. With this in mind, we can then begin to search for an enzyme that is acting to modify the end of cleaved *Bip1*, or purify the *Bip1* mRNA and use mass spectrometry to identify any covalent modifications.

We hope that this search for the enzyme that modifies *Bip1* will shed light on how the *Bip1* mRNA is different from the other mRNAs in fission yeast that are cleaved upon ER stress. How is the *Bip1* mRNA selected for stabilization? Furthermore, why evolve this elaborate mechanism to cleave the *Bip1* mRNA and subsequently rescue it? We have shown the cleavage of *Bip1* can be eliminated with a single point mutation, yet instead the elaborate mechanism to cleave *Bip1*, remove its polyA tail, and then stabilize the mRNA has been maintained. What advantage does the cell derive from doing this? Further understanding of the mechanism behind *Bip1* cleavage and stabilization should help us answer these important mechanistic and evolutionary questions.

We have observed that in addition to its unique stabilization, *Bip1* is translated without a polyA tail. This goes against our current understanding of eukaryotic translation. How does this occur and why is it advantageous for the cell? While it has been shown in budding yeast that the polyA tail is dispensable for translation when the RNA decay machinery is defective (Searfoss and Wickner, 2000), we have observed this
under conditions where the RNA surveillance pathways are thought to be fully functional. Interestingly, when \emph{SKI2} is deleted in fission yeast, we observe a UPR growth defect. A deletion of \emph{SKI2} in budding yeast does not have a similar UPR phenotype. In fission yeast this would presumably cause a stabilization of Ire1 cleaved mRNAs. As these mRNAs would not be subject to rapid degradation, they are still available to be translated. The resulting proteins, synthesized off of truncated mRNAs, would be translocated into the ER, which would compound the ER stress. While this makes sense in fission yeast, there may be a deeper connection between the UPR and the RNA degradation machinery as links between Ski2 and the UPR have appeared in multiple instances.

**Dominant Suppressor of \emph{trl1-100}**

Yet another link between the UPR and RNA degradation became evident when a mutation in Ski2 was identified as a suppressor mutation of \emph{trl1-100}, the UPR deficient allele of tRNA ligase. The identification of this mutation was fraught with difficulty. Many attempts were made to clone any of the nine extragenic suppressors of \emph{trl1-100} that were isolated in the screen (Gonzalez, 2003). Seven of these mutations are dominant and presented themselves as attractive tools to stabilize RNA in any cell, yet standard genetic techniques were unable to identify the mystery mutations. One, and only one, of these genes was finally identified when a genome-wide high throughput technique was utilized to sequence the entire genome (Rubio, 2010).

The mutation was found in Ski2, a component of the heterotrimeric Ski2/Ski3/Ski8 complex that primes mRNA destined for degradation by the 3’-5’ RNA decay pathway. The mutation mapped to one of the conserved RecA domains of the helicase portion of Ski2 and is in a prime location to make direct contact with RNA. The ski2 mutant is not acting solely in the UPR, but is stabilizing all mRNAs in the cell. Most likely, the ski2 mutant is able to suppress the \emph{trl1-100} UPR specific growth defect by stabilizing the cleaved \emph{HAC1} exons. A truncated form of the Hac1 transcription factor can be produced from the stabilized exon and is sufficient to support survival during ER stress. While it is somewhat clear how this mutation is rescuing the UPR defect, it is unclear how this mutation acts to break the 3’-5’ degradation pathway and globally
stabilize mRNAs. Little is known about the mechanisms of Ski2 function. Therefore, this mutation may tell us more about the mechanism of RNA degradation than the mechanism of the UPR.

**Ribosome Mapping of rpl17a and ∆srp101 Mutants**

Genome wide ribosome mapping allows us to determine ribosome positioning with high resolution. It is an amazing way to study translation and how translation is regulated to control gene expression. The knowledge of the exact position at which a ribosome stalls promises to reveal the features of genes, specific mRNA sequence or mRNA secondary structure that influence gene expression.

We attempted to use this technique to examine ribosome pausing in yeast through two different mutant strains: rpl17a and ∆srp101. Rpl17a is the yeast homolog of the *E. coli* ribosomal protein L22 that has been shown to interact with nascent polypeptide in the exit tunnel to regulate gene expression. Srp101 one of the two yeast SRP receptor subunits, SRα, that participates in releasing SRP from signal sequences, transferring the ribosome nascent chain complex to the translocon and relieving the transient translational stall induced by SRP. In both cases, we hoped to identify genes that utilize ribosome pausing as a means of regulation in yeast.

These experiments promise to yield interesting results and would greatly benefit from being repeated. In the short time since they were performed, the ribosome footprinting protocol has evolved and the high throughput sequencing technology has advanced. It is highly likely that the use of cycloheximide on these samples caused artifacts in the data that are hindering our analysis. Additionally, better bioinformatic analysis is greatly needed to decipher the pausing of ribosomes on a positional basis.

**Translational Regulation of HAC1**

Much of my time was spent in pursuit of unlocking the secrets to the translational repression of the HAC1 mRNA. The block to translational initiation of the HAC1 mRNA through the binding of the inhibitory intron to the 5’ UTR has been well established (Rüegsegger et al., 2001). However, published evidence indicated that ribosomes were still bound to the mRNA, yet no protein is made (Chapman and Walter, 1997;
Rüegsegger et al., 2001). My continued efforts to further study the translational repression of the *HAC1* mRNA did not prove fruitful. However, this does not mean that efforts to pursue this avenue of study further would not be successful.

A few lines of evidence suggested that the unspliced *HAC1* (*HAC1*°) mRNA was loaded with translationally silent ribosomes. Initially I attempted to repeat the experiment done by Chapman and Walter, 1997 where they were able to immunoprecipitate an HA-tagged *HAC1*-ribosome nascent chain complex and detect the *HAC1* mRNA. Presumably the HA-tag had been translated, was emerging from the ribosome and was available for immunoprecipitation. The interpretation of these results was that ribosomes were loaded and had begun translating the *HAC1*° mRNA, but were stalled during elongation. I was unable to repeat this experiment, though I was able to pull down the *HAC1* mRNA after UPR induction, which served as a wonderful internal control for the experiment. The remaining evidence for ribosome occupancy on *HAC1*° was that the mRNA co-migrated with heavy polysomes (Rüegsegger et al., 2001). This experiment was performed at the incredibly high magnesium concentration of 30mM. I was also able to observe co-migration of *HAC1*° with polysomes on polysome profiles under these same conditions. However, repeated ribosome profiling of yeast strains under non-UPR conditions failed to reveal ribosome occupancy on *HAC1*° (Ingolia et al., 2009)(chapter 5). Taken together, there is increasing evidence that the translational block seen on *HAC1* is simply a block to initiation imposed by the intron basepairing to the 5’UTR.

A block to initiation is still a very unique and interesting mechanism that deserves further study. The basepairing interaction between the 5’ UTR and the intron has never been dissected carefully. A series of simple experiments would be to mutate the basepairing interaction in the *HAC1* splicing reporter and measure the kinetics of splicing by flow cytometry.

More interestingly, I attempted many times with various techniques to directly immunoprecipitate the *HAC1* mRNA. I tagged the mRNA with the RAT tag (Hogg and Collins, 2007), MS2 sites (Brodsky and Silver, 2000) and a U1A tag (Aragón et al., 2008; Williams and Hall, 1996). While my efforts were hindered by the low abundance of *HAC1* mRNA, as technology and techniques improve this may become a much more
feasible task. Once the mRNA is purified, it can be examined directly for the presence of ribosomes or other RNA binding proteins.

I was also very interested in using the SHAPE technique to determine the secondary structure of the *HAC1* mRNA *in vivo* (Merino et al., 2005). However, while I had *in vivo* control experiments looking at the structure of the 18S rRNA in yeast working well, I was able to show that the *in vivo* concentration of *HAC1* was not high enough to get a signal from *HAC1* for SHAPE experiments. Once the *HAC1* mRNA can be immunoprecipitated and thus concentrated, this technique can easily be applied for structure determination of the mRNA.
References


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