INVESTIGATING THE COMBINED BURDEN OF TRANSCRIPTION AND QUALITY CONTROL ERRORS IN YEAST

by

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Submitted to the Graduate Faculty of the

Kenneth P. Dietrich School of Arts and Sciences

in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

DIETRICH SCHOOL OF ARTS AND SCIENCES

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The Rtf1 subunit of the Paf1 complex is required for specific conserved transcription-coupled histone modifications, including histone H2B lysine 123 monoubiquitylation. In *Saccharomyces cerevisiae*, deletion of *RTF1* is lethal in the absence of Rkr1, a ubiquitin-protein ligase involved in the destruction of nonstop proteins, which arise from mRNAs lacking stop codons or translational read-through into the poly(A) tail. To understand the combined requirement for H2B ubiquitylation and protein quality control in yeast, I performed a transposon-based mutagenesis screen to identify suppressors of *rtf1Δ rkr1Δ* lethality. I found that mutations in the genes encoding RNA Polymerase III subunit Rpc17, sister chromatid cohesion protein Chl1, or the protein chaperone Hsp104 could rescue viability. Of these genes, the role of Hsp104 in yeast is best understood. Hsp104 plays a role in prion propagation, including the maintenance of [*PSI*$^+$], which contributes to the synthesis of nonstop proteins. I demonstrate that *rtf1Δ* and *rkr1Δ* are synthetically lethal only in the presence of [*PSI*$^+$]. The deletion, inactivation, and overexpression of *HSP104* or the overexpression of prion-encoding genes *URE2* and *LSM4* clear [*PSI*$^+$] and rescue *rtf1Δ rkr1Δ* lethality. In addition, the presence of [*PSI*$^+$] decreases the fitness of *rkr1Δ* strains. I investigated whether the loss of *RTF1* exacerbates an overload in nonstop proteins in *rkr1Δ* [*PSI*$^+$] strains but, using reporter plasmids, found that *rtf1Δ* decreases nonstop protein levels, indicating that excess nonstop proteins may not be the cause of synthetic lethality.

Although the mechanism of suppression is not yet clear, mutations in *CHL1* suppress this *rtf1*Δ-specific defect by increasing nonstop protein levels. In addition, I have performed experiments to identify substrates and interacting partners for Rkr1 and these results have further indicated a role of Rkr1 in protein quality control. My data suggest that the loss of Rtf1-dependent histone modifications increases a burden on quality control in *rkr1Δ* [PSI^{+}] cells. Importantly, my research indicates an essential connection between these conserved processes of transcription and quality control.

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PREFACE

My experiences as a graduate student in the Department of Biological Sciences were defined by the individuals I had the pleasure of working with. First, I'd like to thank my mentor and advisor, Dr. Karen Arndt, whose innate ability to teach and explore science I have admired since my first year. Thank you for leading me to this genetic journey, teaching me the tools, and importantly, for letting me find my own way through the unfamiliar but exciting paths it took us. Thank you especially for being someone that I could always discuss the twists and turns of both science and life.

I have been fortunate to work with many wonderful and intelligent scientists, many of whom I am also lucky to call friends. There are too many to account for individually in this space. I'd specifically like to thank all my Arndt lab-mates past and present, as well as members of the Martens and VanDemark labs for all their helpful comments and suggestions over the years and for being the family I needed away from home. My committee members, Drs. Joseph Martens, Jeffrey Brodsky, Andrew VanDemark, and Patricia Opresko, have also been an invaluable collection of ideas during my graduate career. In addition, I have had the privilege of being part of an amazing group of graduate students and faculty that have significantly made this experience what it was. Finally, I would like to thank my family and friends, many of whom I don't see often enough, for never letting me lose sight of the important things in life.

1.0 INTRODUCTION

My dissertation research investigates the combined necessity of the transcription factor Rtf1 and the quality control ubiquitin ligase, Rkr1. Rtf1 is a member of the Paf1 complex, which is necessary for proper gene expression and formation of RNA Polymerase II transcripts through several of its functions, such as its role in histone modifications and the recruitment of 3'-end processing machinery. Rkr1 was originally identified as a protein required for cell viability in the absence of *RTF1*, and has since been shown to be involved in the quality control of nonstop proteins. I have discovered that strains lacking *RKR1* are further burdened by the presence of the prion [*PSI*$^{+}$] and the absence of Rtf1-mediated histone modifications. Because my thesis research is based primarily on investigating the interactions between these components, this introduction chapter will focus on the functions of the Paf1 complex in transcription, the role of Rkr1 as a ubiquitin ligase, and the identification and consequences of prions in yeast.

1.1 TRANSCRIPTION

Transcription by RNA Polymerase II (RNA Pol II) occurs primarily in three stages: initiation, elongation, and termination. As RNA Pol II proceeds through an open reading frame, it encounters obstacles in the form of chromatin structure. In this section, I will discuss how RNA Pol II overcomes these obstacles and proceeds through the transcription cycle.

1.1.1 Transcription occurs within the context of chromatin

The cell employs several mechanisms to navigate through a repressive chromatin environment. These mechanisms include nucleosome remodeling, the exchange of canonical histones for histone variants, and histone modifications. I will briefly describe these mechanisms, with particular detail paid to the mechanisms influenced by the Paf1 complex (further described in section 1.2).

1.1.1.1 Chromatin structure

The repeating unit of chromatin structure is nucleosomes. Nucleosomes are an octamer of two each of four histone proteins (H2A, H2B, H3, and H4), around which are wound approximately 147 bp of DNA. The linker histone H1 further condenses chromatin between nucleosomes. Nucleosomes aid in packaging negatively-charged DNA into chromatin by making DNA contacts with positively-charged histone residues, thereby allowing a large genome to fit into a small nuclear space. Histones are assembled on DNA by histone chaperones, which deposit an H3-H4 tetramer followed by two H2A-H2B dimers to form the higher-order structure (reviewed in (POLO and ALMOUZNI 2006)). The nucleosomal core forms a globular structure that, together

with flexible N-terminal histone tails, serve as sites of binding and modification for proteins that protect or alter this chromatin structure (reviewed in (RANDO and CHANG 2009)). Because nucleosomes also serve as an obstacle to DNA-related processes, histones are dynamically removed, replaced, and modified to allow access to the machinery needed for replication, repair, recombination, and transcription (See the following sections in 1.1.1 for more details).

Several nucleosome positions in the genome are precisely placed, such as the -1 and +1 nucleosome flanking the promoter, while nucleosomes are generally more loosely positioned as they progress through the open reading frame. The rigid polydA/dT rich sequences of promoters help maintain this region nucleosome-free (NFR) *in vivo* and *in vitro* (KAPLAN *et al.* 2009; LEE *et al.* 2007; YUAN *et al.* 2005). Nucleosome-depleted regions have also been identified at the 3' ends of some genes. These NFRs could be due to the presence of anti-sense transcripts at these loci or sites of termination-factor binding, but also to areas of gene-looping, where the 5' and 3' ends of genes interact to keep the transcription cycle active (MAVRICH *et al.* 2008).

Histone genes are essential and extremely conserved. In yeast, two copies of each core histone gene exist: H2A (*HTA1* and *HTA2*), H2B (*HTB1* and *HTB2*), H3 (*HHT1* and *HHT2*) and H4 (*HHF1* and *HHF2*). Genes for H2A/H2B and H3/H4 are paired at divergent loci. Several other copies of histone genes exist in higher eukaryotes. These genes are under cell cycle control and are most highly transcribed during S-phase. Histone gene transcript levels are also highly regulated by degradation and 3'-end processing (reviewed in (MARZLUFF *et al.* 2008)). In humans, histone gene transcripts contain a stem-loop structure at their 3' ends instead of the canonical poly(A) tail of other mRNAs, which is most likely important to the export and degradation of these transcripts in a cell-cycle specific manner (reviewed in (MARZLUFF 2005)).

Yeast histone-encoding mRNAs are polyadenylated, but differentially than other mRNAs, which may help in their regulation (BEGGS *et al.* 2012).

1.1.1.2 Nucleosome remodeling factors and histone chaperones

Nucleosomes must be remodeled or exchanged in order to allow RNA Pol II access to DNA during transcription, as well as other processes. Using ATP-hydrolysis, nucleosome remodeling complexes translocate nucleosomes by breaking DNA-histone contacts. Several classes of nucleosome remodeling factors exist, including the SWI-SNF, ISWI, CHD, and INO80/SWR classes. Distinctions within these families of chromatin remodelers lies within or outside their ATPase domain which results in their differential ability to slide, eject, space, or assemble nucleosomes (reviewed in (CLAPIER and CAIRNS 2009)). The chromatin remodeler Chd1 is an example of a chromatin remodeling enzyme that physically associates with elongation factors such as Rtf1 of the Paf1 complex during active transcription and is required for full nucleosome occupancy at actively transcribed genes (LEE *et al.* 2012; SIMIC *et al.* 2003). In addition, histone modifications can alter the affinity of chromatin remodelers for their substrates. Such is the case for H4 acetylation, which can decrease the activity of Chd1 but increase the activity of RSC (FERREIRA *et al.* 2007). RSC is a type of SWI-SNF family remodeler which is required for RNA Pol II elongation at stress-activated genes and contains bromodomains that can recognize acetylated lysines (KASTEN *et al.* 2004).

Histone chaperones remove and replace histones within nucleosomes to make way for elongating RNA Pol II in an ATP-independent manner. For example, FACT (*fa*cilitates *c*hromatin *t*ranscription), a complex consisting of Spt16 and Pob3, helps open chromatin structure by displacing H2A/H2B dimers (BELOTSERKOVSKAYA *et al.* 2003). The chaperone Spt6 is required for restoring H3/H4 dimers, a process required to occlude RNA Pol II from

recognizing cryptic initiation start sites (BORTVIN and WINSTON 1996; KAPLAN *et al.* 2003). Histone chaperones usually bind directly to histones to aid in remodeling or exchange. By binding to positively-charged histones, chaperones are able to block interactions with unwanted proteins or negatively-charged DNA so that chromatin rearrangement can occur, often with the help of ATP-dependent chromatin remodelers (reviewed in (HONDELE and LADURNER 2011)). It is therefore likely that both histone chaperones and ATP-dependent chromatin remodelers work together with RNA Pol II to remove and reassemble nucleosomes across open reading frames. For example, full activity of the RSC complex *in vitro* is only achieved with the aid of histone chaperones such as Nap1 (LORCH *et al.* 2006).

1.1.1.3 Histone variants

The exchange of the canonical histones H2A, H2B, H3, or H4 for histone variants allows chromatin to be specialized for a particular process. By containing different sequences than the original histones, variants may be differentially modified post-translationally and recruit different factors to chromatin. Many histone variants have been identified across species, some being cell-type specific, and the number of variants for each histone increases with higher eukaryotes. For example, five variants have been identified for H3, including H3.3 in *Drosophila* and mammals and CenH3 which is conserved from yeast through humans (reviewed in (BOYARCHUK *et al.* 2011)). While H3.3 and CenH3 are both associated with marking centromeres, CenH3 has also been correlated with high levels of histone turnover in yeast as well as DNA breaks in humans (LEFRANCOIS *et al.* 2009; ZEITLIN *et al.* 2009).

One of the most important histone variants in transcription is the H2A variant Htz1 (yeast), also known as H2A.Z in humans. H2A.Z has been mapped to centromeres, telomeres, promoters, and gene bodies. As it relates to transcription, it is most often associated with the

nucleosomes flanking the nucleosome-free region of the promoter, where it may help in poising a promoter for activation (RAISNER *et al.* 2005; ZHANG *et al.* 2005a). Structural analysis of H2A.Z suggests that incorporating this variant may destabilize the nucleosome, due to effects of its C-terminal region, which is highly divergent from canonical H2A (FAN *et al.* 2002; SUTO *et al.* 2000). The H2A.Z C-terminal region most likely aids in recruiting specific factors to chromatin that are not recruited in the presence of H2A-containing nucleosomes, either by serving as a binding site for new factors or by opening the nucleosome to facilitate access to DNA and promote transcription. H2AZ is incorporated into nucleosomes through the ATP-dependent SWR1 complex, which binds to H2AZ and exchanges it for H2A within nucleosomes (KROGAN *et al.* 2003a; MIZUGUCHI *et al.* 2004). This complex is also aided by the histone chaperone Chz1 (LUK *et al.* 2007).

1.1.1.4 Histone modifications

Histones are subject to an array of post-translational modifications that mark them for both transcription-dependent and independent processes and help define the chromatin landscape into sections such as active transcription, silenced genes, telomeres, centromeres, and sites of DNA damage. Several types of modifications exist on different residues of each histone, most on the exposed N-terminal lysine-rich histone tails. These modifications define the "histone code," which allows the binding of specific factors to these marks (reviewed in (LEE *et al.* 2010)). In this section, select pathways of histone ubiquitylation, methylation, and acetylation as they relate to transcription will be further discussed.

Histone acetylation, often at multiple lysine residues, correlates with active transcription across open reading frames, most likely aiding to recruit RNA Pol II (DURANT and PUGH 2006; POKHOLOK *et al.* 2005). This dynamic mark is thought to loosen contacts between DNA and

histones by neutralizing the lysine charge, thereby making it more accessible to transcriptional regulators, although the modification may also serve as a binding site for proteins (reviewed in (VERDONE *et al.* 2005)). Histone acetyltransferase (HATs) and histone deacetylase complexes (HDACs) are responsible for placing and removing this mark, respectively. There are several identified eukaryotic HATs, categorized into families such as Gcn5-related, MYST, and p300/CBP (reviewed in (VERDONE *et al.* 2005)). Gcn5, a subunit of the SAGA and ADA complexes, is one well characterized HAT which is required for the correct expression of many genes. It can acetylate both histone H3 and H2B and non-histone co-activator substrates to influence gene regulation (GRANT *et al.* 1997; HOLSTEGE *et al.* 1998; IMOBERDORF *et al.* 2006). Although there are many other HATs, Gcn5 and Esa1, which acetylates H4 and H2A, are responsible for the majority of genome-wide histone acetylation in yeast (DURANT and PUGH 2006).

There are several co-dependencies of histone acetylation and methylation, which are prime illustrations of "histone cross-talk," where one modification influences another. One example involves acetylation and H3 K36 methylation, which is catalyzed by the methyltransferase Set2 at the 3' ends of genes (DROUIN *et al.* 2010; GOVIND *et al.* 2010; KIZER *et al.* 2005; KROGAN *et al.* 2003b). The HDAC Rpd3s complex is recruited to open-reading frames as a result of H3 K36 di-methylation in order to deacetylate nucleosomes. This coordination of events helps prevent cryptic initiation at hidden promoters within genes due to high levels of activating acetylation (CARROZZA *et al.* 2005; KEOGH *et al.* 2005; LI *et al.* 2007b).

Histone H2B is monoubiquitylated on K123 in yeast and K120 in humans by the ubiquitin conjugase and ubiquitin ligase pair Rad6 and Bre1 (HWANG *et al.* 2003; KIM *et al.* 2005; ROBZYK *et al.* 2000; WOOD *et al.* 2003a). This modification is associated with active

transcription on many genes and disrupts higher-order chromatin compaction *in vitro*, but has also been associated with a repressive function on open reading frames, indicating a dual role for this mark (CRISUCCI and ARNDT 2011a; FIERZ *et al.* 2011; MUTIU *et al.* 2007; XIAO *et al.* 2005). H2B K123 monoubiquitylation is a prerequisite for two independent and conserved downstream histone modifications on H3, K4 methylation by Set1 (of the complex COMPASS) and K79 methylation by Dot 1 on active open reading frames (DOVER *et al.* 2002; POKHOLOK *et al.* 2005; ROGUEV *et al.* 2001; SUN and ALLIS 2002; VAN LEEUWEN *et al.* 2002).

Methylation can exist on lysines in a mono, di or tri- state, which may help recruit specific binding factors. Different levels of this histone methylation modification may simply be due to levels of methyltransferase recruitment or to the recruitment of histone demethylases to reverse this mark. For example, H3 K4 tri-methylation is found on active genes while di-methylation can be found across the genome (BERNSTEIN *et al.* 2002; SANTOS-ROSA *et al.* 2002). Set1 is recruited to transcribed loci by Ser-5 phosphorylation on the RNA Pol II CTD (further described in section 1.1.2.1) and therefore H3 K4 tri-methylation is enriched at promoters of active genes. H3 K4 di-methylation is enriched within the open reading frame (NG *et al.* 2003b; POKHOLOK *et al.* 2005). While H3 K4 tri-methylation recruits the histone acetyltransferase complex NuA3, di-methylation specifically recruits the histone deacetylase complex Set3 (KIM and BURATOWSKI 2009; TAVERNA *et al.* 2006). H2B K123 deubiquitylation by the enzymes Upb8 and Ubp10 reverses H2B K123 ubiquitylation and controls its levels in a locus-specific fashion. Ubp8 targets H2B K123 ubiquitylation at the 5' ends of genes where H3 K4 tri-methylation is highest, and Ubp10 targets H2B K123 ubiquitylation associated with H3 K79 methylation within gene bodies (SCHULZE *et al.* 2011).

Histone modifications are regulated by several factors. An important pathway for several of the modifications discussed in this section is through the Paf1 complex (Paf1c). This pathway begins with the cyclin-dependent kinase and regulatory partner Bur1-Bur2, which is recruited to chromatin with the aid of Ser5 phosphorylation of the RNA Pol II CTD (QIU *et al.* 2009). Bur1/Bur2 phosphorylates the C-terminal domain of the elongation factor Spt5, an event that is required for Spt4-Spt5-mediated recruitment of the Paf1c (LIU *et al.* 2009; ZHOU *et al.* 2009). Accordingly, the loss of Bur1-Bur2 also causes a decrease in Paf1c recruitment to chromatin (LARIBEE *et al.* 2005). Paf1c, as well as the upstream factors, are required for H2B K123 ubiquitylation and H3 K4 and K79 methylation (Figure 1) (NG *et al.* 2003a; TOMSON *et al.* 2011; WARNER *et al.* 2007; WOOD *et al.* 2003b; ZHOU *et al.* 2009). Interestingly, Bur1 is also required independently for efficient H2B ubiquitylation through its phosphorylation of Rad6 (WOOD *et al.* 2005). Further, purified yeast Bre1 physically interacts with Paf1 *in vitro*, suggesting a physical link between this pathway and H2B ubiquitylation *in vivo* (KIM and ROEDER 2009). In another related pathway, both Bur1-Bur2 and Paf1c are required for full levels of H3 K36 tri-methylation and recruitment of Set2 to active genes (Figure 1) (CHU *et al.* 2007; KROGAN *et al.* 2003b).

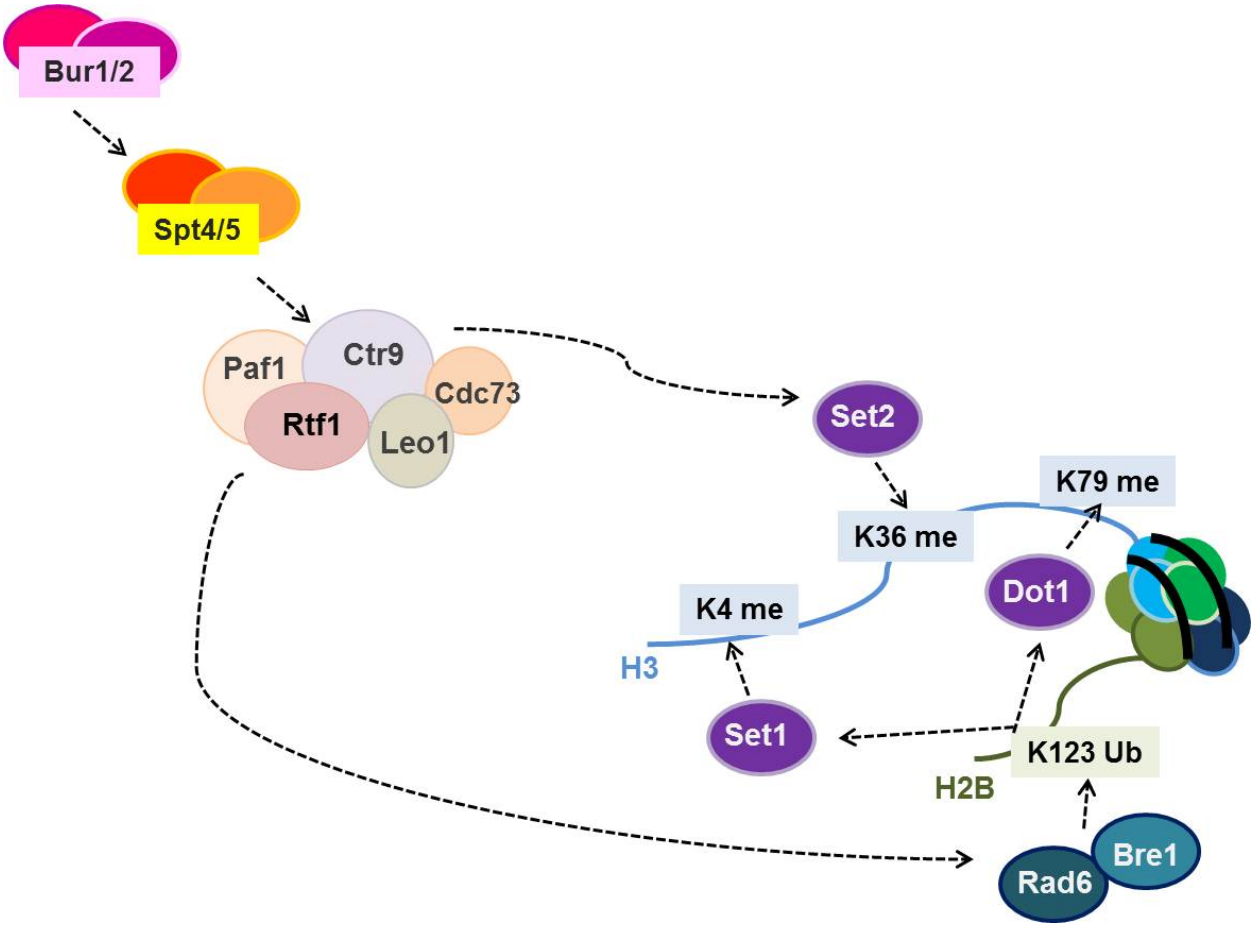

Figure 1: Regulation of histone H2B ubiquitylation and H3 methylation

Histone H2B ubiquitylation by Rad6/Bre1 is a pre-requisite for H3 K4 and H3 K79 methylation by the methyltransferases Set1 and Dot1, respectively (DOVER *et al.* 2002; POKHOLOK *et al.* 2005; ROGUEV *et al.* 2001; SUN and ALLIS 2002; VAN LEEUWEN *et al.* 2002). These marks are regulated by a pathway involving Bur1/2 recruitment to active genes and phosphorylation of Spt5. The Spt4/5 complex in turn recruits the Paf1 complex, which is required for these modifications (NG *et al.* 2003a; TOMSON *et al.* 2011; WARNER *et al.* 2007; WOOD *et al.* 2003b; ZHOU *et al.* 2009). As a separate event, H3 K36 tri-methylation by Set2 also requires a functional Bur1/2-Paf1 complex pathway (CHU *et al.* 2007; KROGAN *et al.* 2003b).

1.1.2 Transcription is a highly regulated process

RNA Polymerase II (RNA Pol II) is a 12 subunit complex that transcribes genes into messenger RNAs, as well as some other noncoding RNAs. RNA Pol II transcription initiates at promoters with the aid of general transcription factors, and proceeds through the DNA template during elongation until exiting during the termination stage. At this point, RNA Pol II is recycled to reenter the transcription cycle. In this section, I will discuss the stages of transcription by RNA Pol II and their regulation.

1.1.2.1 RNA Polymerase II C-terminal domain phosphorylation

The carboxyl-terminal domain (CTD) of the largest RNA Pol II subunit, Rpb1, contains a sequence of repeated heptapeptides ($Y_1S_2P_3T_4S_5P_6S_7$) whose post-translational modifications are essential to transcription regulation (reviewed in (BURATOWSKI 2009)). There are 26 heptapeptide repeats in yeast and 52 in humans. Although several residues are modified in the CTD, CTD serine phosphorylation is particularly well-studied in correlation with the stage of transcription across the open reading frame. These phosphorylation states allow the dynamic recruitment of different transcription and processing factors dependent on the CTD code (reviewed in (BURATOWSKI 2009)). An unphosphorylated CTD recruits initiation factors, for example, while a differently phosphorylated CTD within the open reading frame helps move RNA Pol II through stages of transcription from initiation to elongation to termination and 3'end processing (reviewed in (EGLOFF and MURPHY 2008)).

Ser5 of the CTD is phosphorylated mainly by the cyclin-dependent kinase Cdk7/Kin28 (human/yeast), and aids in recruitment of factors to cap the 5' end of the newly transcribed RNA (CHO *et al.* 1997; RODRIGUEZ *et al.* 2000; SCHROEDER *et al.* 2000). Ser5-P levels decrease as

RNA Pol II moves towards the 3' ends of genes, where Ser2-P levels are highest (CHO *et al.* 2001; KIM *et al.* 2010a). Ser2 is phosphorylated largely by P-TEFb (Cdk9)/Ctk1 (human/yeast), but also by other kinases such as Bur1 (LIU *et al.* 2009; PATTURAJAN *et al.* 1999; PETERLIN and PRICE 2006). This modification helps RNA Pol II transition from transcription elongation to termination and 3' end formation at the end of open reading frames (reviewed in (BURATOWSKI 2009)). Both Ser5-P and Ser2-P are regulated by dephosphorylation by the phosphatases Ssu72, Rtr1 and Fcp1 in yeast, as RNA Pol II moves through the transcription cycle (CHO *et al.* 2001; KRISHNAMURTHY *et al.* 2004; MOSLEY *et al.* 2009). Although this pattern holds true for most genes, the levels of Ser2-P are overall decreased on shorter, non-coding RNA genes, which could explain differences in termination pathways at these loci (TIETJEN *et al.* 2010). In addition to Ser2 and Ser5 phosphorylation, Ser7 has recently been shown to be phosphorylated throughout open reading frames by the kinases Cdk7 and Cdk9, where it highly overlaps with Ser5-P (GLOVER-CUTTER *et al.* 2009; KIM *et al.* 2010a; ST AMOUR *et al.* 2012). Ser7-P has been implicated in helping recruit P-TEFb for Ser2 phosphorylation, and can also be dephosphorylated by Ssu72 (BATAILLE *et al.* 2012; CZUDNOCHOWSKI *et al.* 2012).

1.1.2.2 Transcription Initiation

The pre-initiation complex (PIC), consisting of RNA Pol II and the general transcription factors, assembles at promoters to begin transcription. The promoter region can be TATA box containing, or TATA-less, and usually contains a nucleosome-free region flanked on either side by a well-positioned nucleosome (KAPLAN *et al.* 2009; LEE *et al.* 2007; YUAN *et al.* 2005). The general transcription factors TATA-binding protein (TBP) of TFIID, TFIIA, and TFIIB find and bind the promoter and recruit TFIIF and RNA Pol II, followed by TFIIE and TFIIH to begin transcription by separating the DNA strands and scanning for the transcription start site

(reviewed in (HAHN and YOUNG 2011)). Co-activators such as Mediator, SAGA, and NuA4 help control which genes are recognized and bound by the PIC. Factors required for efficient transcription initiation require proper CTD phosphorylation. For example, the general transcription factor TBP and co-activator Mediator complex are both recruited to unphosphorylated CTD to start the transcription cycle, and are released upon Ser5 phosphorylation (MAX *et al.* 2007; USHEVA *et al.* 1992).

1.1.2.3 Transcription Elongation

As RNA Pol II proceeds through the open reading frame and Ser5 becomes phosphorylated, a new set of factors is recruited to properly synthesize the nascent RNA. This transition involves histone modifying enzymes, histone variants, histone chaperones, and chromatin remodeling enzymes, which help RNA Pol II navigate a repressive chromatin structure by loosening histone-DNA contacts and disassembling nucleosomes (See section 1.1.1 of this Introduction for a more detailed review of these processes). RNA splicing and processing factors are also recruited during transcription elongation through association with the CTD (reviewed in (SELTH *et al.* 2010)). Ser5-P is required for recruitment of the 5' end capping machinery and the Set1 H3 K4 methyltransferase through Paf1c (reviewed in (EGLOFF and MURPHY 2008)). As Ser2 is phosphorylated, histone modifying enzymes such as the H3 K36 methyltransferase Set2 associate with the CTD, also with the help of Paf1c (KIZER *et al.* 2005; KROGAN *et al.* 2003b). Several mechanisms are employed by these factors to facilitate RNA Pol II transcription. For example, H2A/H2B dimers, more frequently than H3/H4, can be displaced and exchanged to make way for elongating RNA Pol II, but RNA Pol II is able to transverse nucleosomes without displacement *in vitro*, indicating that this might not be necessary at all genes (reviewed in (SELTH *et al.* 2010)) (THIRIET and HAYES 2005).

1.1.2.4 Transcription Termination and RNA 3'-end processing

As elongating RNA Pol II reaches the end of the DNA template, it recruits several factors to terminate transcription and process the nascent mRNA. This step is crucial in completing gene expression, as improper 3'-end processing can result in errors in exporting or translating the transcript, as well as target the transcript for premature quality control and degradation (reviewed in (MANDEL *et al.* 2008)).

Transcription termination at RNA Pol II –transcribed genes can occur via two pathways (reviewed in (KUEHNER *et al.* 2011)). The first involves recognitions and cleavage of the nascent mRNA, followed by polyadenylation. Transcripts extend beyond the translational stop codon in a region called the 3' untranslated region (or UTR), which can be anywhere from 50 to thousands of nucleotide base pairs in length (reviewed in (PROUDFOOT 2011)). Within this region exists a cleavage and polyadenylation signal, often the conserved sequence AAUAAA, which is required for 3'end polyadenylation of nascent transcripts by a poly(A) polymerase, such as Pap1 in yeast (reviewed in (ZHAO *et al.* 1999)) (WICKENS and STEPHENSON 1984). This signal and others are recognized by the cleavage and polyadenylation specificity factor (CPSF) and cleavage stimulatory factor (CstF) in mammals, factors which are conserved in higher eukaryotes (reviewed in (PROUDFOOT 2011). In addition, one transcript can contain multiple, alternate polyadenylation sites, which can aid in regulating transcript levels through the formation of different 3' UTRs (reviewed in (PROUDFOOT 2011)). Transcription is required for proper RNA 3' end processing, as the RNA Pol II CTD recruits the cleavage and polyadenylation factors and this event is necessary for the release of RNA Pol II from newly transcribed mRNA (reviewed in (KUEHNER *et al.* 2011)). The cleavage and polyadenylation factor Pcf11, for example, directly

and preferentially interacts with Ser-2 phosphorylation on the CTD to promote transcription termination (LICATALOSI *et al.* 2002; MEINHART and CRAMER 2004)

The second method of transcription termination for RNA Pol II transcripts exists mainly for noncoding, nonpolyadenylated RNAs such as snRNAs and snoRNAs (reviewed in (KUEHNER *et al.* 2011)). This process requires the Sen1 DNA helicase and the RNA binding proteins Nrd1 and Nab3 in yeast, as well as the nuclear exosome (TRAMP) in order to cleave and trim the 3' end (STEINMETZ *et al.* 2001; STEINMETZ *et al.* 2006). Interestingly, the non-polyadenylation factor Nrd1 also interacts with Ser5-P and Ser7-P CTD to process specific transcripts, indicating the importance of RNA Pol II in coordinating this termination activity (MEINHART and CRAMER 2004; TIETJEN *et al.* 2010; VASILJEVA *et al.* 2008). In general, it has been hypothesized that the shorter ncRNA genes have a different distribution of CTD phosphorylation, consisting mostly of Ser5-P and Ser7-P, which helps distinguish which transcription termination pathway to employ (reviewed in (KUEHNER *et al.* 2011)) (GUDIPATI *et al.* 2008).

1.2 THE PAF1 COMPLEX MODULATES SEVERAL EVENTS IN TRANSCRIPTION

The Paf1 complex (Paf1c) physically associates with elongating RNA Pol II to facilitate transcription. In this section, I will describe the known functions of the Paf1 complex, including its roles in transcription elongation, histone modifications, and 3'-end formation of nascent transcripts (Figure 2).

1.2.1 Paf1c is an elongation complex

There are several factors that aid in the proper transcription of a chromatin template. Several studies support the role of Paf1c as one such factor that is specifically required for proper transcription by elongating RNA Pol II. This complex is conserved from yeast through humans, though with slightly different members. In *Saccharomyces cerevisiae*, Paf1c consists of the five proteins Paf1, Ctr9, Leo1, Cdc73, and Rtf1 (KROGAN *et al.* 2002; MUELLER and JAEHNING 2002; SHI *et al.* 1997; SQUAZZO *et al.* 2002). In humans, the Paf1 complex can exist as hCtr9, hLeo1, hCdc73, hPaf1, and hSki8, sometimes with hRtf1 depending on purification strategies (KIM *et al.* 2010b; ROZENBLATT-ROSEN *et al.* 2005; ZHU *et al.* 2005)

Genetic studies showed that the loss of *RTF1* was synthetically lethal with several mutations in genes connected to transcription elongation-related processes, such as the mediator subunit *SRB5*, CTD kinase *CTK1*, CTD phosphatase *FCP1*, and FACT chromatin remodeling subunit *POB3* mutations (COSTA and ARNDT 2000). Further data supporting Paf1c in transcription elongation include physical interactions between Paf1c and the elongation factors Spt4-Spt5 and the Spt16-Pob3/FACT complex, as well as phenotypes associated with transcription elongation defects, such as sensitivity to 6-azauracil (SQUAZZO *et al.* 2002). In humans, RNA Pol II and the transcription elongation factor SII/TFIIS have also been shown to physically and directly interact with Paf1c to enhance transcription elongation (KIM *et al.* 2010b). *In vitro*, reconstituted or purified hPaf1c can stimulate and enhance transcription of a DNA template in a dosage dependent manner (KIM *et al.* 2010b). Depletion of Paf1c subunits also causes a decrease in transcription elongation both *in vivo* and *in vitro* (RONDON *et al.* 2004; TOUS *et al.* 2011). Additionally, the loss of Paf1c members causes delayed histone removal in the *GAL1* coding region upon induction, and a decrease in nucleosomal occupancy across the

SER3 promoter, indicating a role for Paf1 in proper nucleosome levels (MARTON and DESIDERIO 2008; PRUNESKI *et al.* 2011).

1.2.2 Paf1c physically associates with RNA Pol II on actively transcribed genes

The namesake of the Paf1 complex was coined for *P*olymerase *a*ssociated *f*actor, as it purifies with RNA Polymerase II (WADE *et al.* 1996). It physically interacts with RNA Pol II in all of its phosphorylated forms (ROZENBLATT-ROSEN *et al.* 2005). Paf1c is distributed across all active open reading frames with transcribing RNA Pol II from the transcription start site to the poly(A) site, but loss of Paf1c does not change Poll II distribution (KIM *et al.* 2004; MUELLER *et al.* 2004). Subunits of the Paf1c bind RNA *in vitro* and *in vivo,* which may also play a role in aiding to localize the complex to actively transcribed genes (DERMODY and BURATOWSKI 2010). A reduction or mutation in Rtf1 or Cdc73 causes loss of Paf1c from chromatin, suggesting their role in tethering the complex to elongating RNA Pol II (AMRICH *et al.* 2012; MUELLER *et al.* 2004; WARNER *et al.* 2007). In coordination with this, loss of *PAF1* causes more severe growth defects and phenotypes. However, loss of *PAF1* also results in a decrease in both Rtf1 and Cdc73 levels, which could explain the defects of *paf1Δ* strains in both reduction of chromatin association in addition to its own phenotypes (BETZ *et al.* 2002; MUELLER *et al.* 2004).

Although Paf1c physically associates with RNA Pol II on all actively transcribed genes, the loss of its members only affect a subset of genes and these effects can be both positive or negative (CRISUCCI and ARNDT 2011a; MAYER *et al.* 2010; SHI *et al.* 1996). In addition, genome-wide studies comparing loss of *PAF1* or *CTR9* show that they have overlapping but not identical effects on transcript abundance (PENHEITER *et al.* 2005).

1.2.3 An effect of Paf1c on CTD phosphorylation

Loss of Paf1c subunits disrupts RNA Pol II phosphorylation of its heptapeptide sequence. For example, studies have shown that the loss of *PAF1*, *CTR9*, or *CDC73* causes a decrease in total Ser2-P levels on the CTD (NORDICK *et al.* 2008b), while others have correlated this CTD phosphorylation defect with a global length of poly(A) tails (MUELLER *et al.* 2004). Loss of Paf1c subunits also causes a decrease in recruitment of factors that require CTD phosphorylation, such as the cleavage and polyadenylation factor Cft1 (NORDICK *et al.* 2008b). Because proper CTD phosphorylation serves as a platform for recruitment of multiple factors, it is likely that changes in CTD phosphorylation or interacting proteins are likely the cause of many phenotypes seen in the absence of *PAF1*.

1.2.4 Histone modifications

One of the best described roles of the Paf1c members is their requirement for specific and conserved histone modifications. These histone modifications have been shown to be important for a variety of processes, indicating their importance in transcription.

1.2.4.1 H2B monoubiquitylation

The Paf1c is required for monoubiquitylation of H2B in eukaryotes, a function mainly attributed to the subunit Rtf1 (NG *et al.* 2003a; TOMSON *et al.* 2011; WARNER *et al.* 2007; WOOD *et al.* 2003b). Rtf1 regulates this modification through an unknown mechanism requiring the ubiquitin conjugating enzyme Rad6 and the ubiquitin protein ligase Bre1 (HWANG *et al.* 2003). Without Rtf1, both yeast and human chromatin also lack the downstream methylation of H3 on K4 and

K79 by the methyltransferases Set1 and Dot1, respectively (DOVER *et al.* 2002; NG *et al.* 2003a; SUN and ALLIS 2002; WOOD *et al.* 2003b).

1.2.4.2 H3 K36 tri-methylation and histone acetylation

Specific members of Paf1c (Paf1, Ctr9, and Cdc73) and the upstream Bur1-Bur2 cyclin-dependent protein kinase, are required for proper histone H3 K36 tri-methylation by the methyltransferase Set2, as well as the proper recruitment of Set2 to active genes (CHU *et al.* 2007; KROGAN *et al.* 2003b). This defect was shown by western analysis of global H3 K36 methylation, as well as chromatin immunoprecipitation at specific genes, which indicated a decrease in H3 K36 tri-methylation. This reduction was greater at the 5' end than the 3' ends of active genes. The same study found a concurrent increase in histone acetylation at the 5'ends of some genes and varying effects on histone acetylation at the 3' ends. However, this increase in histone acetylation at the 5' end was not dependent on the decrease in methylation (CHU *et al.* 2007) .

1.2.5 Role of Paf1c in RNA 3'-end formation

The correct 3'-end formation of RNAs is important to creating stable, functional RNAs that can be properly exported and translated. The Paf1 complex is involved in regulating several aspects of transcription termination and polyadenylation. Errors in this process may lead to changes in transcript levels and targeting by quality control pathways.

1.2.5.1 Cleavage and polyadenylation

Both yeast and human Paf1c are required for the correct maturation of RNA Pol II transcripts (MUELLER *et al.* 2004; NAGAIKE *et al.* 2011; NORDICK *et al.* 2008a; PENHEITER *et al.* 2005; SHELDON *et al.* 2005). This complex is required for proper mRNA cleavage, polyadenylation, and export to the cytoplasm of transcripts produced from a reporter construct in human cells (NAGAIKE *et al.* 2011). The human ortholog of Cdc73 is also required for proper cleavage and formation of the 3' ends of histone mRNAs, resulting in their stabilization (FARBER *et al.* 2010). Processed human histone mRNAs are normally not polyadenylated, which aids in regulating the levels of these transcripts (HARRIS *et al.* 1991; STAUBER and SCHUMPERLI 1988). hCdc73 also physically associates with the 3' cleavage and polyadenylation specificity factor CPSF and the cleavage stimulation factor CstF, confirming an important role for this factor in coordinating 3'-end formation (ROZENBLATT-ROSEN *et al.* 2009).

In yeast, loss of Paf1c members results in decreased poly(A) tail length and alternative poly(A) site usage (MUELLER *et al.* 2004; STRAWN *et al.* 2009). This may be due to a decrease in the recruitment of 3'-end processing factors, as the cleavage and polyadenylation factor Cft1 requires Rtf1 and Cdc73 for proper association with Ser5-P of the CTD. In addition, Ctr9 also physically interacts with Cft1 (NORDICK *et al.* 2008b). Paf1c is also important in preventing premature transcription termination by Sen1/Nab3/Nrd1 at the open reading frame *FKS2* in response to cell stress (KIM and LEVIN 2011). Spt5 is required for recruitment of both the RNA cleavage factor CFI and Paf1c, further connecting this complex in many facets of transcription termination on RNA Pol II genes (MAYER *et al.* 2012). Interestingly, Paf1c also functions in termination outside of RNA Pol II transcription, as it is also required for efficient rRNA processing (PORTER *et al.* 2005; ZHANG *et al.* 2009).

1.2.5.2 snoRNA 3'-end formation

Members of the Paf1 complex are also required for the efficient 3'-end formation of non-mRNA transcripts, such as non-polyadenylated small nucleolar RNAs (snoRNAs). This function is at least partially dependent on Paf1-mediated histone modifications such as H2B ubiquitylation (SHELDON *et al.* 2005; TOMSON *et al.* 2011). The proper 3'-end formation of snoRNAs requires the proteins Nab3 and Nrd1, the Sen1 helicase, and cleavage/polyadenylation complex factors, as well as the 3' to 5' activity of the exosome (ALLMANG *et al.* 1999; FATICA *et al.* 2000; MORLANDO *et al.* 2002; STEINMETZ *et al.* 2001). Regulation of snoRNA transcription termination by Paf1 most likely occurs through Nab3 and Nrd1, as deletion of *PAF1* affects Nrd1 recruitment to these genes (SHELDON *et al.* 2005).

1.2.5.3 mRNA quality control of Paf1-regulated transcripts

Improperly processed transcripts resulting from loss of Paf1c, such as those described above, can be substrates for mRNA quality control pathways (PENHEITER *et al.* 2005) and (reviewed in (CRISUCCI and ARNDT 2011b; JAEHNING 2010)). For example, loss of *PAF1* causes altered usage of poly(A) sites on the genes *SDA1* and *MAK21*. The resulting extended transcripts are recognized as substrates by the nonsense-mediated decay machinery and are targeted for degradation (PENHEITER *et al.* 2005). Therefore, changes in transcripts in *paf1Δ* cells may be due not only to decreased transcription, but also to errors in 3'-end processing that lead to the destruction of aberrant mRNAs. Likewise, changes in 3'-end processing may not affect transcript levels, but instead affect their ability to be exported or translated. This is the case for the yeast nonsense allele *ade1-14*, which requires a functional Paf1c for proper levels of protein. The *ade1-14* transcript levels are unchanged in mutant strains. Instead, it seems that these Paf1c

mutant strains use a different poly(A) site, which results in decreased export or translational expression of the transcript (STRAWN *et al.* 2009).

In human cells, hPaf1 has been isolated with the additional subunit hSki8, which is a member of the SKI complex necessary for proper mRNA degradation (ZHU *et al.* 2005). In yeast, this complex also contains Ski2 and Ski3 and is cytoplasmic, but in humans this complex is both cytoplasmic and nuclear, where it interacts with the exosome to promote 3' to 5' mRNA decay (BROWN *et al.* 2000; ZHU *et al.* 2005). The nuclear exosome associates along transcriptionally active open reading frames in *Drosophila*, suggesting that like Paf1c, it also plays a role in transcription elongation (ANDRULIS *et al.* 2002). Knockdown of hSki8 results in decreased levels of other Paf1c members and several associated histone modifications, similar to the loss of hPaf1c (ZHU *et al.* 2005). The finding that human Paf1c stably associates with a member of an mRNA decay complex implies that mRNA surveillance occurs co-transcriptionally, and further suggests a Paf1c role in targeting mRNA quality control and decay of aberrant transcripts.

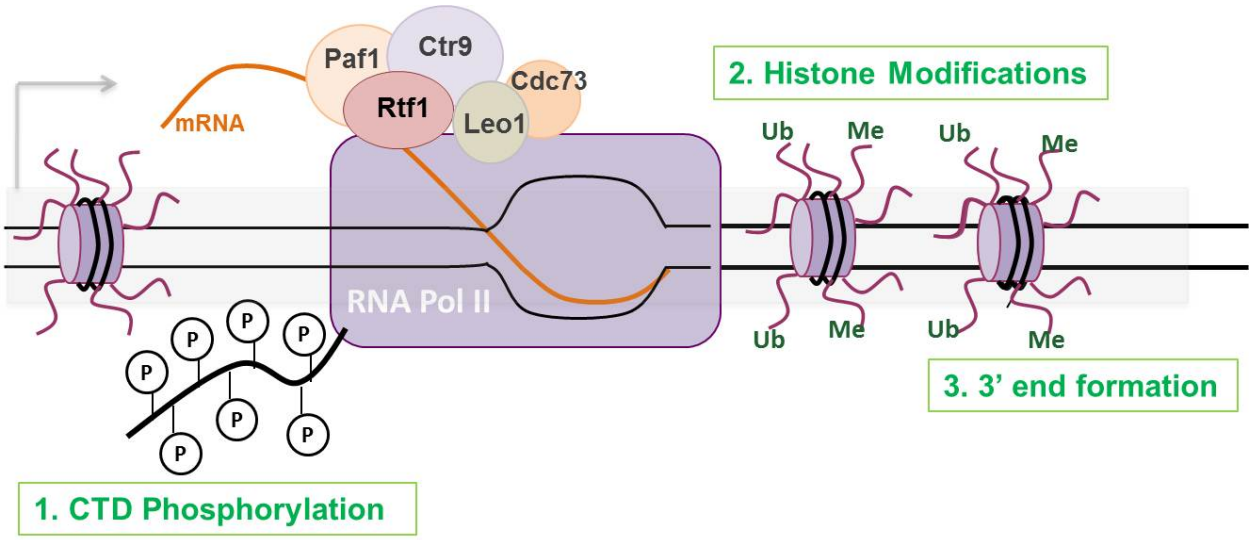

Figure 2: The roles of the Paf1 complex

The Paf1 complex plays several roles in transcription through its physical association with elongating RNA Pol II on all actively transcribed open reading frames and its ability to bind RNA (Complex as shown is representation, and does not necessarily reflect physical associations between members). These conserved functions include maintaining proper CTD phosphorylation, histone modifications such as H2B K123 ubiquitylation and H3 methylation, as well as 3' end formation of nascent transcripts (reviewed in (CRISUCCI and ARNDT 2011b; JAEHNING 2010)).

1.2.6 Roles of Paf1c outside RNA Pol II transcription

The Paf1c has been connected to other processes in addition to proper transcription elongation, histone modification, and mRNA formation. These functions may be due indirectly to its association with RNA Pol II, but also to other unidentified roles. For example, mutations in Paf1c members are synthetically lethal with a mutation in the cell cycle regulator genes *SWI4* and *SWI6*, and affect the expression of many cell cycle associated genes (Costa and Arndt unpublished data, (BETZ *et al.* 2002; PORTER *et al.* 2002)). Paf1c is also required for DNA damage repair in a Rad26-dependent pathway as well as in global genomic repair through H2B K123 ubiquitylation. The same study also showed that, in cooperation with the elongation factors Spt4 and Spt5 in yeast, Paf1c suppresses a Rad26-independent transcription-coupled repair pathway (TATUM *et al.* 2011). Outside its physical connection to RNA Pol II, Paf1c is also required for efficient RNA Pol I transcription and associates with rDNA without affecting Pol I occupancy (ZHANG *et al.* 2009).

Recently, the Paf1 complex has also been associated with chromosome segregation. The methyltransferase for H3 K4me, Set1, has also been shown to methylate the kinetochore protein Dam1. Dam1 is also phosphorylated by the Aurora kinase Ipl1, and both Dam1 phosphorylation and methylation are important for its function in chromosome segregation (ZHANG *et al.* 2005b). Further investigation into Set1 regulation of this process uncovered the necessity for the Paf1 complex and Rad6-Bre1, as well as H2B K123 ubiquitylation, for Set1-mediated Dam1 methylation, connecting the Paf1 complex to mitosis and suggesting that histone cross-talk can occur outside the context of chromatin (LATHAM *et al.* 2011).

In higher eukaryotes, both Leo1 and Cdc73 have been implicated in Wnt signaling, which can affect both cell fate and cancer (MOSIMANN *et al.* 2006). hCdc73 overexpression leads to an

increase in the expression of Wnt target genes through mediating Wnt translocation into the nucleus. In addition, *Drosophila* Cdc73 and Leo1 directly bind the Wnt signaling regulator β-catenin (MOSIMANN *et al.* 2006).

1.2.7 Connections of Paf1c to disease

Due to the many functions of the Paf1 complex, mutations or overexpression of the genes encoding these subunits can have a myriad of effects on human disease. hPaf1 is overexpressed in pancreatic cancer cells, and the overexpression of hPaf1 can transform nononcogenic cells, suggesting this protein is an oncogene (MONIAUX *et al.* 2006). hCdc73, also known as parafibromin, is often mutated in hyperparathyroidism-jaw tumors (CARPTEN *et al.* 2002).The overexpression of the gene encoding parafibromin, HRPT2, has also been associated with liver, pancreatic, and breast cancers (CHANG *et al.* 2005; PARADA *et al.* 1998; STANGE *et al.* 2006)

In addition, human Paf1C-mediated histone modifications have been associated with disease. Mistakes in these histone modifications can lead to erroneous transcription and result in cancer. For example, reduced H2B ubiquitylation results in decreased expression of the p53 tumor suppressor gene and increased expression of tumor-promoting genes (SHEMA *et al.* 2008). Paf1c-mediated histone modifications can also be exploited in an infection. A strain of influenza encodes a protein that mimics the H3 K4 methylation sequence and binds to Paf1c, thereby resulting in decreased gene expression, including genes required for antiviral immunity (MARAZZI *et al.* 2012).

1.3 RKR1 UBIQUITYLATION AND PROTEIN QUALITY CONTROL

Rkr1 (*R*ing domain mutant *k*illed by *rtf1*) is a ubiquitin ligase originally identified in a screen for factors required for viability in the absence of the transcription factor Rtf1 (BRAUN *et al.* 2007). It possesses a RING domain, which has ubiquitin ligase activity *in vitro* (BRAUN *et al.* 2007). Rkr1 is required for the quality control of nonstop proteins, resulting from translational read-through into the poly(A) tail of mRNA (BENGTSON and JOAZEIRO 2010). In this section, I will further discuss the process of protein ubiquitylation in yeast, with particular focus on what is known about Rkr1.

1.3.1 The components of ubiquitylation

Ubiquitin is a conserved 76 amino acid protein that covalently is attached to other proteins to target them for processes such as apoptosis, cell cycle progression, DNA repair, and proteasome-mediated degradation (reviewed in (WEISSMAN 2001)). The ubiquitylation pathway consists of three main steps. The first occurs when a ubiquitin activating enzyme (E1) activates ubiquitin, and the next occurs as the E1 transfers ubiquitin from its active-site cysteine to an active-site cysteine in a ubiquitin-conjugating enzyme (E2). The third step occurs when the E2 transfers ubiquitin to a lysine residue on a substrate, targeted with the aid of a ubiquitin ligase (E3) (Figure 3) (reviewed in (DESHAIES and JOAZEIRO 2009)). Ubiquitin is usually attached to a substrate lysine via an isopeptide bond with its C-terminal glycine. The substrate can then targeted for its indicated pathway based on number and placement of ubiquitin proteins. This dynamic process is reversible, as deubiquitylating enzymes can remove ubiquitin from substrates (reviewed in (LOVE *et al.* 2007)).

1.3.1.1 Ubiquitin activating enzymes

In yeast, only one ubiquitin activating enzyme, or E1 (Uba1) exists, and this protein is essential for cell viability (MCGRATH *et al.* 1991). Early studies with a temperature-sensitive allele of a mammalian E1 verified the importance of this enzyme to protein turnover (CIECHANOVER *et al.* 1984; FINLEY *et al.* 1984). E1s are ATP-dependent enzymes which first bind to MgATP and then form a high-energy thioester between its active-site cysteine and the carboxyl-terminal glycine of a free ubiquitin (reviewed in (FANG and WEISSMAN 2004)). An E1 can bind two ubiquitin molecules at one time, one at its active site and one as an adenylate intermediate that can be donated to the active site once it is free (HAAS and ROSE 1982). The E1 can transfer the activated ubiquitin to the active-site cysteine of an E2 (reviewed in (FANG and WEISSMAN 2004)).

1.3.1.2 Ubiquitin conjugating enzymes

E2s contain a conserved ubiquitin-conjugating catalytic (UBC) fold which contains a cysteine residue that binds activated ubiquitin from the E1 (BURROUGHS *et al.* 2008). There are 11 conserved ubiquitin E2s in yeast (Ubc1-8, Ubc10, Ubc11, and Ubc13), while humans have over 30 (reviewed in (VAN WIJK and TIMMERS 2010)). Most E2s are found both in the nucleus and cytoplasm, although several are associated with organelles, such as the case for Ubc6/7, which resides on the surface of the endoplasmic reticulum (BIEDERER *et al.* 1997).

1.3.1.3 Ubiquitin ligases

E3s allow for specificity in substrate selection in the ubiquitin pathway. The predominant classes of E3 proteins are HECT and RING domain ubiquitin ligases, although E3s without these domains have been described, including the Skp1-Cul1-F box protein (SCF) ubiquitin ligases

and U box proteins (reviewed in (ARDLEY and ROBINSON 2005)). HECT E3s contain a 350 amino acid-terminal domain containing a conserved cysteine that forms a thioester with ubiquitin (HUIBREGTSE *et al.* 1995). Many HECT E3s also contain an N-terminal WW domain, which participates in interactions with proline-rich proteins, and is most likely important in targeting substrates (reviewed in (WEISSMAN 2001)). The F box protein of an SCF complex recognizes the specific substrate to be recruited for ubiquitylation by the complex (reviewed in (ARDLEY and ROBINSON 2005). RING domain (Really Interesting New Gene) ubiquitin ligases represent a class of proteins that number in the hundreds in yeast and humans. This RING domain is described as a cysteine-rich motif, with the canonical sequence Cys-X-Cys-X-Cys-X-His-X-Cys-X-Cys-X-Cys-X (where X is a number of any amino acids), although other variations exist (FREEMONT *et al.* 1991). Two atoms of zinc are bound by the cysteine and histidine core of this domain, which serves as a rigid domain for protein-protein interactions (BARLOW *et al.* 1994). Not all proteins with RING domains possess ubiquitin ligase activity alone, but most RING E3s studied so far have been shown to have activity if not alone, then in conjunction with a second E3 partner. For example, the breast cancer associated ubiquitin ligases BRCA1 and BARD1 have little activity *in vitro*, yet robust activity as a heterodimer (HASHIZUME *et al.* 2001). NMR studies with BRCA1 have shown that the RING domain of this E3 is essential to its interaction with its E2 partner, a finding which appears will be expanded to more E2-RING E3 complexes (BRZOVIC *et al.* 2003; ZHENG *et al.* 2000).

RING domain and U box E3s do not directly ubiquitylate a substrate, but rather serve as a platform for the E2-Ub complex to come in close proximity with the substrate by binding both substrate and E2 at the same time (reviewed in (ARDLEY and ROBINSON 2005). The ubiquitin is then transferred from the E2 to the substrate before the E2 detaches and recharges with another

ubiquitin. This is in contrast to HECT domain E3s, which do acquire ubiquitin from their E2 partner to form an E3-Ub thioester as an intermediate to substrate ubiquitylation (Figure 3) (reviewed in (DESHAIES and JOAZEIRO 2009)).

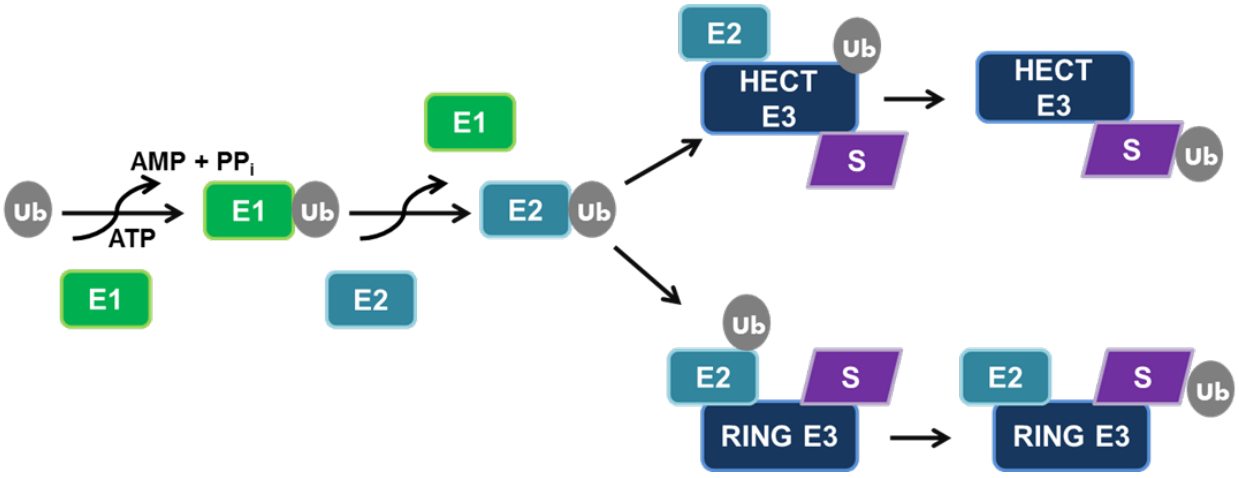

Figure 3: The ubiquitylation pathway.

In an ATP dependent step, the ubiquitin activating enzyme, E1, binds ubiquitin via a thioester bond. Next, the activated ubiquitin is transferred to a ubiquitin conjugase enzyme or E2. This E2 then binds a partner E3, or ubiquitin ligase, for ubiquitylation of a specific substrate. A HECT domain E3 accepts transfer of ubiquitin from the E2 before ubiquitylating the substrate directly. A RING domain E3, however, does not directly interact with ubiquitin, but rather mediates the transfer of ubiquitin from an E2 to the substrate (adapted from (WEISSMAN 2001)).

1.3.2 Interactions and outcomes of the ubiquitylation pathway

The ubiquitylation pathway described above results in a modified substrate now targeted for a specific process. As new studies emerge on the interactions between the components involved in this pathway, they paint a picture of a very complicated network where specificity is achieved by a precise partnership of proteins and amino acid residues. In this section, I will describe some of these interactions during ubiquitylation and how they result in such a myriad of consequences.

1.3.2.1 Interactions among ubiquitin components

The components of the ubiquitin pathway must work in faithful succession, which relies on the specific order and interactions of these proteins. For example, in order to polyubiquitylate a substrate, an E2 must obtain a new ubiquitin molecule. The E2 would therefore shuttle between the E1 to bind ubiquitin and the E3 to aid in transfer of the ubiquitin to a substrate, and is unable to bind both E1 and E3 at the same time (ELETR *et al.* 2005). As may be expected for a reaction that is part of a multi-step pathway, the E2-E3 interaction is often not stable, and this transient interaction is therefore difficult to detect. To make this interaction increasingly difficult to predict, the known interacting residues are not conserved between E2-E3 pairs and are likely specific to the E2-E3 partnering involved (reviewed in (DESHAIES and JOAZEIRO 2009)). In addition, ubiquitin ligases have been identified for which multiple E2 partners work in succession – with one E2 initiating a slow substrate ubiquitylation and a different E2 elongating a faster addition of a ubiquitin chain, such as the case with APC/C in yeast (RODRIGO-BRENNI and MORGAN 2007). Because it is difficult to capture E2-E3 interactions, few structural studies

have been done. However, recent studies have illuminated potential E2-E3 partners through large-scale screens for interactions in human proteins using a yeast 2-hybrid assay (MARKSON *et al.* 2009; VAN WIJK *et al.* 2009).

1.3.2.2 Ubiquitin lysine residues target substrates for a specific process

A large-scale proteomics study in yeast identified over 1000 proteins ubiquitylated at any time, with ubiquitin chains being formed via any of its seven conserved lysines (6,11,27,29,33,48, and 63) in yeast, indicating that there is a large diversity in polyubiquitin chains (PENG *et al.* 2003). This site specific linkage seems to be characteristic mostly of the E2 involved, and it is possible that a RING E3 can therefore use multiple E2s to create branched ubiquitin chains (KIM *et al.* 2007). That said, one E2 can mediate different linkages depending on the E3 with which it interacts, such as human E2 UbCH5 mediating Lys6, 11, 48, or 63 linkages with different RING E3s (reviewed in (DESHAIES and JOAZEIRO 2009)). Reactions involving HECT E3s, however, can direct their own chain linkage with a specific ubiquitin lysine residue independent of its E2 partner (WANG and PICKART 2005).

The different ubiquitin chain linkages aid in directing ubiquitylated proteins to different cellular processes. For example, K63 chains are indicative of a signaling process, such as DNA repair (SPENCE *et al.* 1995) and endocytosis (reviewed in (HICKE 2001)) while K48 and K11 chains are targets to the proteasome (THROWER *et al.* 2000; XU *et al.* 2009). The finding that a ubiquitin can form chains via any linkage or combination of linkages, implies that the complexity of ubiquitin-targeting is yet to be fully understood (PENG *et al.* 2003; WEISSMAN *et al.* 2011).

1.3.2.3 Polyubiquitylation and proteasome-targeting

Due to translation or folding mistakes, nearly 30% of all translated proteins are targeted for degradation immediately following synthesis in mammalian cells to allow recycling of defective proteins and generation of small peptides (SCHUBERT *et al.* 2000; WHEATLEY *et al.* 1982). At least four ubiquitins linked by their Lys48 residues are required for proteasome-targeting via the proteasome subunit Rpn10 and Rpn13 (CHAU *et al.* 1989; DEVERAUX *et al.* 1994; HUSNJAK *et al.* 2008). The 26S proteasome has a 19S regulatory complex (composed of a six ATPase base and lid) at either end of a 20S core, which recognizes a multi-ubiquitin chain (reviewed in (KLOETZEL 2001)). Substrates targeted for proteasome-mediated degradation are deubiquitylated by the 19S subunit Rpn11 (VERMA *et al.* 2002), unfolded, and channeled into the 20S core, which is comprised of four stacks of α and β subunits (reviewed in (KLOETZEL 2001)). The α subunits form the outer rings, which accept substrates and releases products. The β subunits, some with catalytically active residues, form the inner rings (GROLL *et al.* 1997; LOWE *et al.* 1995). These β subunits are responsible for degrading and recycling substrates into smaller peptide chains and amino acids (GROLL *et al.* 1997).

1.3.2.4 Substrate monoubiquitylation

In addition to polyubiquitylation at various lysines, substrates can be monoubiquitylated. Monoubiquitylation usually acts as a cellular signal, and has been connected with multiple processes, including transcription, endocytosis, and viral replication (reviewed in (HICKE 2001)). For example, monoubiquitylation of histone H2B K123 by the E2 Rad6 and E3 Bre1 in yeast is a well-studied and conserved mark of active transcription (HWANG *et al.* 2003). This H2B mono-ubiquitylation signal is a prerequisite for downstream histone H3 methylation (DOVER *et al.* 2002; SUN and ALLIS 2002). The linker histone H1 is monoubiquitylated in *Drosophila*

embryos, another example of an important transcription-related cell signaling mark (PHAM and SAUER 2000). Several plasma membrane proteins, such as G-protein-coupled receptors, must also be monoubiquitylated in order to be internalized and degraded by the lysosome (reviewed in (HICKE 1999)).

How a substrate is selectively targeted for monoubiquitylation rather than polyubiquitylation is unknown, but factors have been identified that give clues to different ways proteins can regulate this event. For example, Rad23 in yeast and humans inhibits the formation of multi-ubiquitin conjugates (ORTOLAN *et al.* 2000). Some multi-ubiquitin chains also require the presence of a factor termed an E4 (KOEGL *et al.* 1999). The presence of deubiquitylating enzymes in the cell also suggests the possibility that ubiquitin chains are trimmed to regulate their lengths (reviewed in (HICKE 2001)).

1.3.2.5 Identification of ubiquitylated substrates

Hundreds of E3s exist in eukaryotes, allowing substrate targeting and specificity of the ubiquitin pathway. Although thousands of proteins have been found to be ubiquitylated (PENG *et al.* 2003), finding a substrate for a particular E3 is a very challenging task. Besides having a very large pool of both E3s and potential substrates, ubiquitylated substrates are often quickly turned over and the interactions between E3 and substrate are therefore transient. Systematic identification of E3 and substrate pairings is a daunting though not impossible, undertaking; therefore several studies have used more global methods of identifying substrates of E3s (reviewed in (KAISER and HUANG 2005)).

Large-scale *in vitro* approaches have been successful in identifying E3 targets. For example, the APC/C E3 complex regulates substrates only during mitosis. *Xenopus* oocyte cDNAs were translated *in vitro* and incubated with lysate from either oocytes in interphase or

mitosis, and the resulting proteins after incubation were compared. Those proteins lost from mitotic lysate incubations were further investigated as targets of APC/C (LUSTIG *et al.* 1997; MCGARRY and KIRSCHNER 1998). Another study involving the BRCA1/BARD1 heterodimer involved immunoprecipitation of the complex and incubating the resulting proteins with FLAG tagged ubiquitin in an *in vitro* ubiquitylation reaction. FLAG-tagged substrates were then identified by mass-spectrometry (SATO *et al.* 2004). In yeast, two studies have been done to identify substrates for the E3 Rsp5 using screens of purified potential substrates and protein microarrays (GUPTA *et al.* 2007; KUS *et al.* 2005).

Ubiquitin profiling has been developed to study ubiquitin substrates *in vivo*. Initially, this technique was performed by expressing His-tagged ubiquitin in cells and performing immunoprecipitation against all His-ubiquitylated proteins under denaturing conditions, which helps prevent degradation or deubiquitylation of substrates. These substrates were identified by mass spectrometry to reveal a picture of all ubiquitylated proteins within yeast at a given time (PENG *et al.* 2003). Ubiquitin profiling has been used for the proteasomal ubiquitin receptor Rpn10 by isolating all ubiquitylated proteins from wild type cells in yeast and comparing it to *rpn10* mutant cells (MAYOR *et al.* 2005). Methods such as these may prove useful in the future for identifying specific E3 targets.

1.3.3 Rkr1 is a quality control ubiquitin ligase

Rkr1 is a conserved RING domain ubiquitin ligase with genetic connections to transcription. Interestingly, it is also required for the quality control of nonstop proteins. In this section, I will describe protein quality control mechanisms and the role of Rkr1 in this process.

1.3.3.1 Quality control ubiquitin ligases

With limited resources on hand, it is important that cells employ mechanisms to overcome stresses in quality control and are able to recycle aberrant proteins. Many proteins are mistranslated or become misfolded after synthesis and are unable to be refolded by the chaperone machinery, leaving them as targets for protein quality control by the ubiquitylation pathway (WHEATLEY *et al.* 1982). There have been several identifications of ubiquitin ligases that target aberrant proteins in yeast and these ligases are localized in various parts of the cell, such as the endoplasmic-reticulum associated E3s Hrd1 and Doa10 (reviewed in (HAMPTON 2002)), the nuclear E3 San1 (GARDNER *et al.* 2005), and the "N-end rule pathway" cytoplasmic E3 Ubr1 (BARTEL *et al.* 1990).

Quality control ubiquitin ligases are specific for their class of substrates, either relying on sequence characteristics of these targets or the help of chaperones to identify them. San1 physically binds to and recognizes abnormal nuclear proteins, but not their normally folded counterparts. ER-associated degradation requires the help of chaperones and other proteins for target recognition (reviewed in (FREDRICKSON and GARDNER 2012)) (GARDNER *et al.* 2005; METZGER *et al.* 2008). In the N-end rule pathway, Ubr1 recognizes potential substrates via an N-terminal sequence which includes a lysine for ubiquitylation (BARTEL *et al.* 1990). The role of Ubr1 in this process is to target short-lived proteins for turnover (reviewed in ((VARSHAVSKY 1997)).

In some cases, quality control ubiquitin ligases have overlapping roles. For example, a study was performed to find factors required for degradation of the misfolded cytoplasmic protein CPY‡ (HECK *et al.* 2010; MEDICHERLA *et al.* 2004), finding that both the N-end rule pathway E3 Ubr1 and nuclear E3 San1 were required for quality control of this protein. This

study revealed two parallel pathways for quality control of misfolded proteins. In the cytoplasm, Ubr1 targeted this substrate and other misfolded proteins with the aide of chaperones in a manner independent from its role in the N-end rule pathway, while San1 ubiquitylated the same substrates in the nucleus after a chaperone-dependent transport step (HECK *et al.* 2010). Therefore, the cell is able to employ several mechanisms to target aberrant proteins, sometimes in parallel approaches. With hundreds of ubiquitin ligases within a cell and thousands of proteins targeted for ubiquitylation, it is likely that many more quality control E3s will be identified in future studies.

1.3.3.2 Rkr1 is involved in nonstop protein quality control

Rkr1 was originally identified as a RING finger ubiquitin ligase required in the absence of the transcription factor, Rtf1, suggesting that these two proteins are most likely required in yeast for parallel processes with a common essential goal (Figure 4) (BRAUN *et al.* 2007). *rkr1Δ* strains also genetically interact with the loss of H2B K123 ubiquitylation, a histone modification modulated by Rtf1, indicating that it most likely functions specifically in parallel to this Rtf1-mediated event. Strains lacking *SPT10*, which functions in histone gene expression, are also lethal in the absence of *RKR1*, further indicating a function genetically connected to transcription (BRAUN *et al.* 2007). The RING domain of Rkr1 (C4HC3 type) was shown to have ubiquitin ligase activity *in vitro*, confirming its role as an E3 (BRAUN *et al.* 2007). Rkr1 is conserved throughout higher eukaryotes, with conservation highest in the N-terminal region and C-terminal RING domain. The human homologue, known as RNF60 or ZNF294, is commonly mutated in colon cancer (IVANOV *et al.* 2007), while the mouse homologue, Listerin, has been implicated in neurodegeneration (CHU *et al.* 2009). In yeast, Rkr1 has also been called Ltn1 to reflect this homology to Listerin (BENGTSON and JOAZEIRO 2010).

Rkr1 is found bound to ribosomes and co-fractionates predominantly with the 60S subunit (BENGTSON and JOAZEIRO 2010; FLEISCHER *et al.* 2006). It has also been shown to interact with the 19S proteasome subunit, indicating it most likely functions to target proteins for degradation (VERMA *et al.* 2000). In agreement with this data, I and others have found this protein to be predominantly, though not exclusively, localized in the cytoplasm of yeast (BENGTSON and JOAZEIRO 2010).

In a screen for mutations that caused an increase in nonstop protein levels in yeast, a deletion of *RKR1* was identified (WILSON *et al.* 2007). Nonstop proteins result from translational read-through into the poly(A) tail, which can happen during translation of both nonstop transcripts (lacking a stop codon), or by translational failure to recognize normal stop codons. Nonstop mRNAs and nonstop proteins are both targeted for degradation (FRISCHMEYER *et al.* 2002; VAN HOOF *et al.* 2002; WILSON *et al.* 2007). Loss of *RKR1* resulted in an increase in nonstop protein levels from a reporter, but not an increase in nonstop mRNA levels (WILSON *et al.* 2007). These results were confirmed by a later study, which showed that Rkr1 was required for the ubiquitylation and degradation of nonstop proteins. This study also showed that Rkr1 recognizes these nonstop substrates by the presence of a poly-lysine chain, which results from translational read-through of the mRNA poly(A) tail (BENGTSON and JOAZEIRO 2010). Rkr1 was also shown to co-immunoprecipitate with a nonstop protein reporter immediately following protein synthesis (BENGTSON and JOAZEIRO 2010). It was therefore proposed that Rkr1 is recruited to the ribosome upon translational-stalling at the poly(A) sequence in order to ubiquitylate the newly synthesized nonstop protein for proteasomal degradation. Although to date, Rkr1 has not been shown to interact with an endogenous nonstop protein, these results strongly suggest that Rkr1 functions in nonstop protein quality control. Additionally, it has

recently been shown that the loss of *RKR1* causes sensitivity to media containing geldanamycin, which inhibits Hsp90 chaperone activity and causes an increase in aggregated and unfolded proteins targeted for degradation (THEODORAKI *et al.* 2012). This phenotype is shared by the loss of other quality control ubiquitin ligases in yeast, such as Ubr1 and San1, and suggests that Rkr1 may have broader implications for quality control (THEODORAKI *et al.* 2012). Understanding why yeast require the efficient degradation of nonstop proteins when faced with the loss of Rtf1 transcription-related functions is the basis of my thesis research (Figure 4).

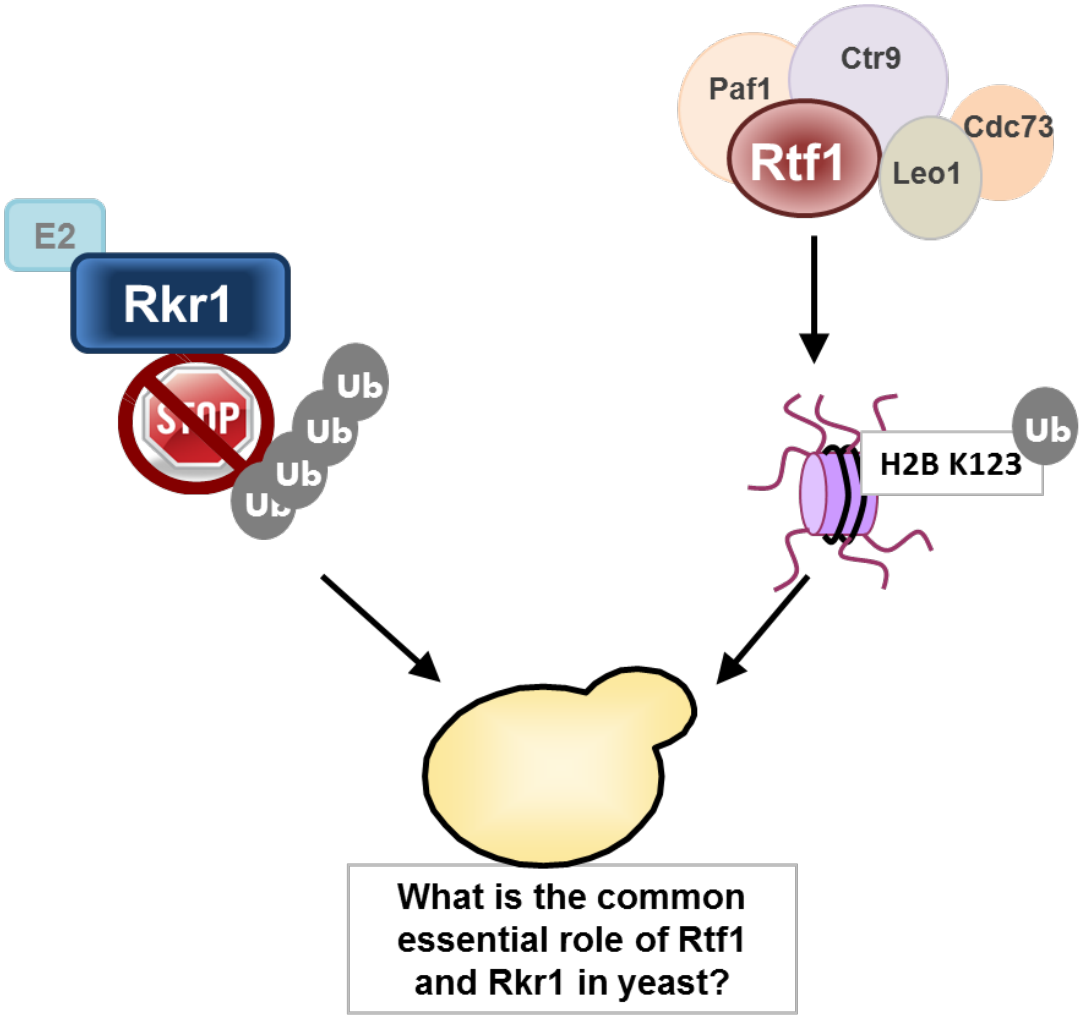

Figure 4: Rkr1 is required in the absence of Rtf1-mediated H2B ubiquitylation.

Yeast can survive the loss of the transcription factor *RTF1*, a member of the Paf1 complex required for H2B K123 ubiquitylation. However, in combination, the loss of *RTF1* and the ubiquitin ligase *RKR1* is lethal in yeast (BRAUN *et al.* 2007). Rkr1 is required for the ubiquitylation and degradation of nonstop proteins, produced from translational read-through into the poly(A) tail (BENGTSON and JOAZEIRO 2010; WILSON *et al.* 2007). This synthetic interaction between *RTF1* and *RKR1* in yeast suggests that the two factors are required for parallel processes that result in a common essential role. Investigation into this interaction is the basis of my thesis research.

1.4 YEAST PRIONS

Prions are infectious protein aggregates found in organisms from yeast to humans. Although these proteins have presumed normal functions as soluble proteins, aggregation can cause a disruption in normal protein function. This, in addition to the nature of having an aggregated protein in the cell, can cause problems in organismal function. Additionally, these protein aggregates can be inherited or transferred to other cells to perpetuate the prions within a population. Yeast as a model organism has served as an important tool for studying prion genesis, phenotypes, and transmission. In my thesis work, I identified the prion [*PSI*$^{+}$] as required for the synthetic lethal relationship between *rtf1Δ* and *rkr1Δ* mutations in yeast. This chapter will further explain the nature of prions in *Saccharomyces cerevisiae*.

1.4.1 Characteristics of a prion

Prions are insoluble infectious proteins which are able to propagate without genetic material, and be transferred to other cells or passed on by non-Mendelian inheritance. Prions, together with chaperones, are also able to recruit soluble proteins around them to further propagate the structure.

1.4.1.1 Prion domains

Only the prion domain is required for successful transmission and seeding of the prion in other yeast cells (MASISON *et al.* 1997; MASISON and WICKNER 1995). The majority of prion domains in yeast are characterized by glutamine and asparagine (Q/N-rich) rich regions which form amyloids, filamentous aggregates of β-sheets which can also be formed *in vitro* (DEPACE *et al.* 1998). These amyloids are proposed to be an in-register parallel β-sheet structure of polar zipper-like structure, where identical residues touch and interact through their side chains (Figure 5A) (reviewed in (WICKNER *et al.* 2011)) (PERUTZ *et al.* 2002; WICKNER *et al.* 2010). This structure recruits new soluble monomers to the prion form by directing the same conformation as the other proteins in the filament (Figure 5B) (reviewed in (WICKNER *et al.* 2011). The order of amino acids in the prion domain are not important for original amyloid formation, and shuffling this domain does not affect propagation (ROSS *et al.* 2004). However, the prion domain sequence itself is important to transmission of a prion, as differences in this sequence can prevent transmission of a prion variant between different yeast species, a phenomena known as the "species barrier" (BATEMAN and WICKNER 2012). Prions have variants which affect their stability, phenotype, and transmission ability, and these variants may be caused by differences in amyloid structures caused by sequence differences or initial differences in filament formation (Figure 5B) (BRADLEY and LIEBMAN 2003; EDSKES *et al.* 2009; SCHLUMPBERGER *et al.* 2001).

In yeast a bioinformatics study was performed to identify proteins with Q/N-rich domains and a low number of hydrophobic or charged amino acids which would hinder the formation of in-parallel amyloids (ALBERTI *et al.* 2009). This study initially identified approximately 200 *S. cerevisiae* proteins which included known yeast prion-forming proteins Sup35 and Rnq1. Several of the top 20 protein domains from this list were verified as having the propensity to form

aggregates, indicating that the characteristics of this domain are indeed important to prions (ALBERTI *et al.* 2009). Another study in yeast determined that prion domains indeed are characterized by a Q/N-rich region, but that there is not an exact requirement for the number of these residues in a region and that a Q/N-rich region did not necessarily dictate formation of a prion (TOOMBS *et al.* 2010). Outside of *S. cerevisiae*, prions have been characterized that do not contain Q/N-regions, such as $[Het\text{-}S]_y$ in the fungus *Podospora anserina* and the human prion protein PrP, indicating that the propensity to form a prion does not absolutely require these residues (BALGUERIE *et al.* 2003).

1.4.1.2 Prion propagation by chaperones

In yeast, molecular chaperones are required for the propagation of all prions studied to date. In particular, prion propagation requires a functional Hsp104 protein, as an *HSP104* deletion or inhibition of this protein through growth of yeast on guanidine hydrochloride (GuHCl) results in the inability to transfer prions to daughter cells (FERREIRA *et al.* 2001; JUNG and MASISON 2001). The role of Hsp104 in yeast is to disaggregate proteins after heat shock (SANCHEZ and LINDQUIST 1990), an activity that requires an ATPase domain and threading of an aggregated substrate through the Hsp104 channel (reviewed in (HASLBERGER *et al.* 2010)). This same activity is responsible for breaking prion amyloids into smaller oligomers, which can then be efficiently passed onto daughter cells. Therefore, Hsp104 is required to create "seeds" with infectious ends that will become the prions of the next generation and recruit additional soluble protein to the prion form (reviewed in (HASLBERGER *et al.* 2010)).

Interestingly, overexpression of *HSP104* cures cells of one identified yeast prion, [PSI^+], but not other prions tested (CHERNOFF *et al.* 1995; MORIYAMA *et al.* 2000) (reviewed in (CROW and LI 2011)). In fact, overexpression of *HSP104* can lead to an increase in other yeast prions

(KRYNDUSHKIN *et al.* 2011). Although the exact mechanism of this unique interaction is unclear, studies have shown that curing cells of [*PSI*$^{+}$] by increased Hsp104 dosage involves a different mechanism than curing by deletion of *HSP104*, which results in an increase in prion-particle size that may then be too large to be transferred to a daughter cell (DERDOWSKI *et al.* 2010).

Additional chaperones in yeast are required for prion propagation besides Hsp104, and these findings present a very complicated network of chaperones involved in this process. For example, the Hsp70 chaperone Ssa1 is also required for the propagation of [*PSI*$^{+}$], and overexpression of *SSA1* can also cure [*PSI*$^{+}$] similar to Hsp104 (MATHUR *et al.* 2009; SONG *et al.* 2005). However, overexpression of Ssa1 in the presence of excess Hsp104 antagonizes [*PSI*$^{+}$] clearing (NEWNAM *et al.* 1999). Ssa1 functions with multiple partners, including the Hsp40 co-chaperones Ydj1 and Sis1 (reviewed in (KAMPINGA and CRAIG 2010)). Sis1 is required for the propagation of several yeast prions, including [*URE3*], [*PIN*$^{+}$], [*PSI*$^{+}$], and [*SWI*$^{+}$] (HIGURASHI *et al.* 2008; HINES *et al.* 2011; SONDHEIMER *et al.* 2001), however overexpression of Sis1 is only known to cure the yeast prion [*SWI*$^{+}$] (HINES *et al.* 2011). Similarly, deletion of *YDJ1* is only known to cure [*SWI*$^{+}$] (HINES *et al.* 2011) while increased dosage of Ydj1 can cure yeast of the prion [*URE3*] (MORIYAMA *et al.* 2000).These studies indicate the importance of chaperones to prion transmission and propagation and also reveal that a very intricate network of proteins is involved in this process. Much additional work is needed to understand how these chaperones and others work together to influence which prions are present at one time in yeast.

1.4.1.3 Prion-prion interactions

Prions also have a profound influence on each other, as the presence of one prion can both negatively and positively affect the presence of other prions. This may be due to the similar structure of prion domains in yeast, as well as their overlapping chaperone requirements

(reviewed in (DERKATCH and LIEBMAN 2007)). The most well-studied example of this interaction is between [*PIN*$^+$] and [*PSI*$^+$]. The prion [*PIN*$^+$] is generally required for the *de novo* appearance of [*PSI*$^+$] (DERKATCH *et al.* 2001; DERKATCH *et al.* 1997). However, weak and strong variants of both [*PIN*$^+$] and [*PSI*$^+$] have been identified, based on phenotype and aggregation, and these variants as well as the levels of these prions can influence whether or not they impair each other's inheritance (BRADLEY and LIEBMAN 2003). This indicates the influence of [*PIN*$^+$] on [*PSI*$^+$] is not a simple event. Additionally, the presence of [*PSI*$^+$] and [*URE3*] can enhance the formation of [*PIN*$^+$] (DERKATCH *et al.* 2001), while [*URE3*] and [*PSI*$^+$] have been shown to negatively affect the appearance of each other especially in the presence of [*PIN*$^+$] (SCHWIMMER and MASISON 2002). The ways in which prions interact with each other will likely be further elucidated, or further complicated, as more yeast prions are being identified and studied.

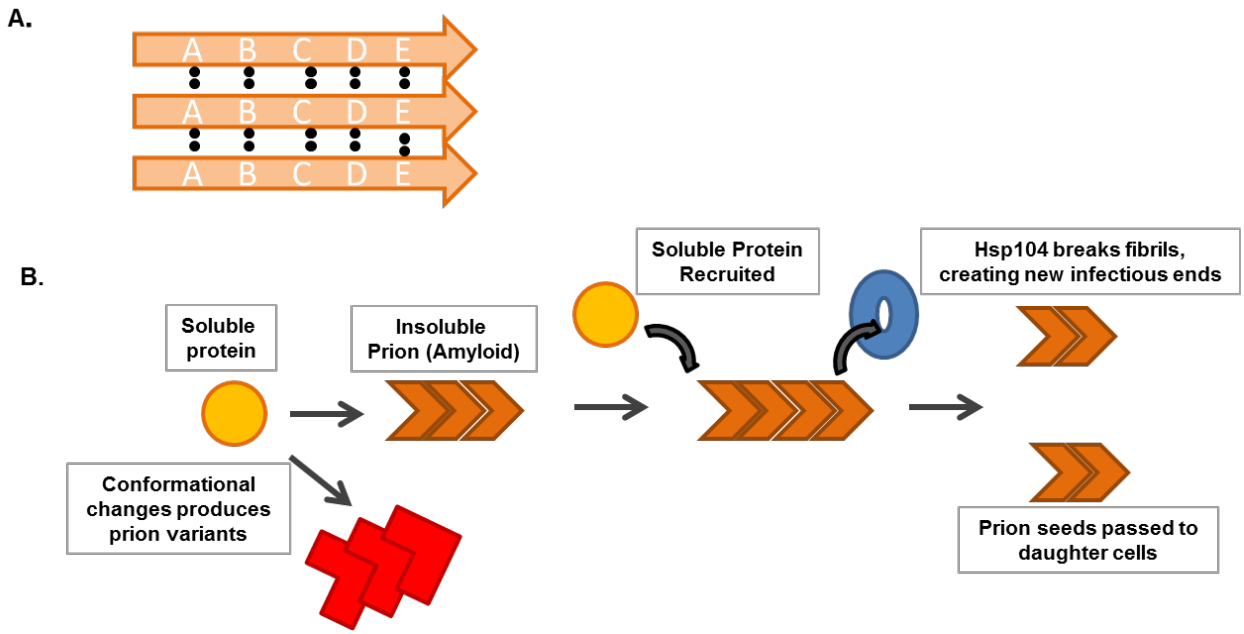

Figure 5: Prion formation in yeast

(A) Q/N-rich domains of prions are proposed to be organized into amyloid fibrils in an in-register parallel β-sheet structure, where identical residues interact through their side chains (reviewed in (WICKNER *et al.* 2011)) (WICKNER *et al.* 2010). (B) Prions are the insoluble form of the native protein, which aggregate to form an amyloid fibril. These amyloids can form *de novo*, and can be arranged into different prion variants which recruit the same variants of soluble protein. Prions are propagated by chaperones, such as Hsp104, which breaks the fibrils into small pieces with new infectious ends that can be passed to daughter cells (reviewed in (WICKNER *et al.* 2011).

1.4.2 Well-studied yeast prions

With the current knowledge of typical prion domains, several studies have emerged over the years to identify and confirm new prions in *S. cerevisiae*. In addition, the continued study of well-known prions has led to better understanding of their resulting phenotypes in yeast.

1.4.2.1 [*PSI*$^+$]

[*PSI*$^+$] was the first identified yeast prion and is the most well-studied. It is the prion form of the translation termination factor Sup35, an essential protein required for proper stop codon recognition. Sup35, also known as translational release factor eRF3, normally functions with eRF1 to release nascent polypeptide chains. When a cell contains Sup35 as [*PSI*$^+$], it results in translational read-through of both normal stop codons and nonsense (premature) stop codons, leading to a nonsense suppression phenotype and production of nonstop proteins (PAUSHKIN *et al.* 1996; WILSON *et al.* 2005). In addition, improper translation termination caused by [*PSI*$^+$] also results in the inability to properly degrade aberrant nonstop or nonsense mRNAs which normally rely on this Sup35 function (WILSON *et al.* 2005). Therefore, the presence of this prion causes multiple identified nonsense suppression phenotypes, although these phenotypes can vary with strain background (TRUE *et al.* 2004). The most common assay to assess for the presence of [*PSI*$^+$] is with an *ade1-14* reporter, which contains a nonsense codon. Soluble Sup35, or a [*psi*$^-$] strain, is able to recognize the early stop codon, producing a nonfunctional Ade1 protein and an Ade$^-$ phenotype. Cells containing [*PSI*$^+$] lead to nonsense suppression and translational read-through of *ade1-14*, producing Ade$^+$ cells (Figure 6) (CHERNOFF *et al.* 1995). [*PSI*$^+$] is the only prion known to be cured by both the overexpression and deletion of *HSP104* (CHERNOFF *et al.* 1995), indicating that this chaperone is needed at precise levels for [*PSI*$^+$] propagation.

1.4.2.2 [*PIN*$^+$]

The prion [*PIN*$^+$] was first identified by its role in [*PSI*$^+$] propagation, and named for [*PSI*$^+$] inducibility (DERKATCH *et al.* 1997). Years later, the gene *RNQ1* was found to encode the prion-forming protein (DERKATCH *et al.* 2001). Although many studies have been done on the prion potential of [*PIN*$^+$] in relation to other yeast prions, to date, there is no known non-prion function for Rnq1. It has also been shown that [*PIN*$^+$] is required to propagate polyQ aggregation in a yeast model of Huntington's disease (MERIIN *et al.* 2002). Therefore, without other phenotypes to attribute to this prion, the presence of [*PIN*$^+$] in yeast is generally determined by the ability of a cell to induce or propagate other prions.

1.4.2.3 [*URE3*]

[*URE3*] is another early-identified yeast prion and has also been used frequently as a model for understanding prion genesis and inheritance. [*URE3*] is the prion form of Ure2, which negatively regulates nitrogen metabolism genes, such as *DAL5*, by sequestering the transcription factor Gln3 to the cytoplasm (COX *et al.* 2000; WICKNER 1994). There are multiple methods for detecting [*URE3*], including using yeast reporters genes fused to a *DAL5* promoter (SCHLUMPBERGER *et al.* 2001).

1.4.2.4 Other identified yeast prions

More recently identified yeast prions include [*SWI*$^+$], [*OCT*$^+$], [*ISP*$^+$], and [*MOT3*$^+$]. Interestingly, all of these prions are encoded by genes that normally function in a transcription-related process. [*SWI*$^+$] is the prion form of the SWI/SNF chromatin remodeling complex factor Swi1 (DU *et al.* 2008). The corepressor Cyc8 is the soluble form of [*OCT*$^+$] (PATEL *et al.* 2009). Sfp1 is a transcription factor that regulates 10% of the yeast genome, and it can form the prion

[*ISP*$^{+}$] (ROGOZA *et al.* 2010). [*MOT3*$^{+}$], encoded by *MOT3*, is the prion form of a transcriptional regulator originally identified as a potential prion from the screen for Q/N-rich domains performed by Alberti et al (ALBERTI *et al.* 2009).

Additional prions have been described by phenotype in yeast, but the genes encoding the prions have yet to be confirmed. Common characteristics of the well-studied prions include the presence of a Q/N-rich domain, the ability to form amyloid fibrils *in vivo* and *in vitro*, the ability to be transferred and inherited, and the dependence on chaperones for propagation. Deletion of the gene encoding the prion should cure cells of the prion over generations, and an increase in prion protein levels should increase the *de novo* appearance of the prion (reviewed in (CROW and LI 2011)). Although over 20 potential yeast proteins have been identified by their Q/N-rich domains (ALBERTI *et al.* 2009), many have yet to be confirmed as true prions with these characteristics. Likely, future studies on these candidates will reveal an even larger pool of prions that could exist in *S. cerevisiae* or be transferred to or within a population.

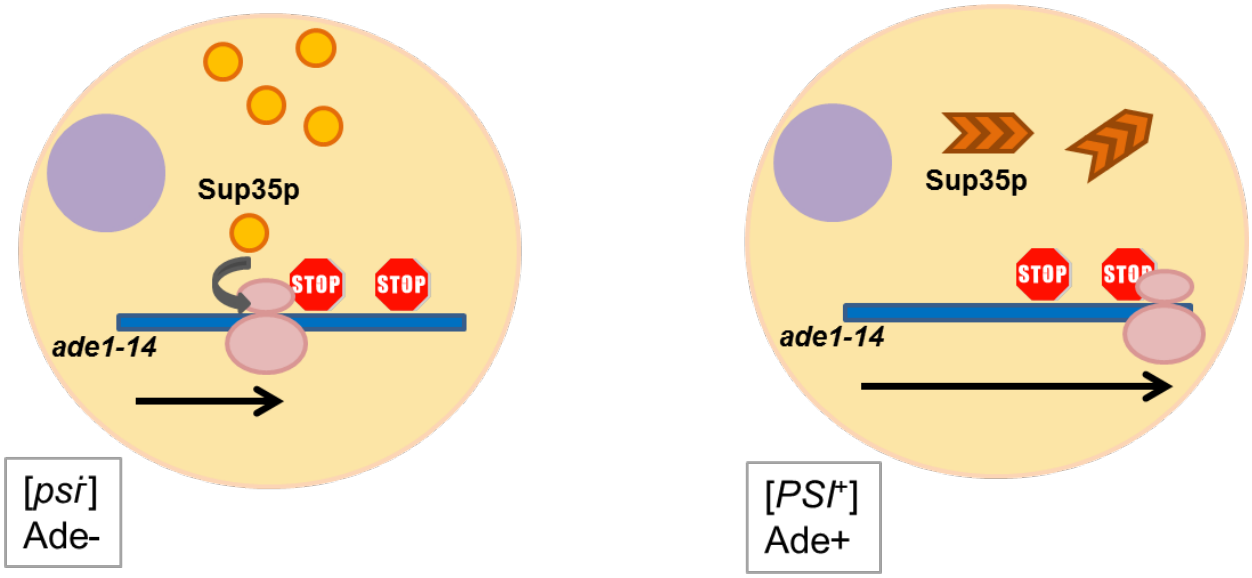

Figure 6: Sup35 aggregation in [*PSI*$^+$] cells causes nonsense suppression

Soluble Sup35 functions in translation termination. During translation of the *ade1-14* reporter in yeast, which contains a premature stop codon, the nonsense codon is recognized by Sup35 and Ade1 protein is not produced, making the cells Ade$^-$. However, in [*PSI*$^+$] cells, aggregated Sup35 cannot efficiently recognize stop codons. This results in translational read-through of the *ade1-14* nonsense codon and production of functional Ade1 protein, making the cells Ade$^+$ (CHERNOFF *et al.* 1995).

1.4.3 Prions in humans

Yeast have served as an excellent model system for studying prion inheritance and transmission. Prions have been found in multiple organisms and learning about them in yeast may prove beneficial in understanding their roles in human disease.

1.4.3.1 PrP

The only identified human prion is called PrP for "prion protein," which can be transmitted to other humans through a genetic mutation causing amyloid formation or by incubation with infectious brain material (HSIAO *et al.* 1989). This one prion protein in humans can cause several known neuronal diseases, such as Kuru, Creutzfeldt-Jakob disease, and Gerstmann-Sträussler-Scheinker syndrome, most likely by forming differently structured aggregates (reviewed in (SOTO and CASTILLA 2004)). Although PrP does not have the same classical Q/N structure as prions identified in yeast, it does form similar fibrils of β-sheets in its insoluble form (PAN *et al.* 1993). The discovery and identification of PrP and its encoding gene has led to the protein-only hypothesis, which is the basis of prion biology from yeast to humans – that only a misfolded protein is needed to infect other organisms, not any genetic material. To date, however, infectious PrP has not been made *in vitro* that can cause disease *in vivo*. Studies in yeast have been more successful at this approach with the prion [*PSI*$^{+}$], as well with understanding how prions are propagated and transferred (reviewed in (HOFMANN *et al.* 2012; SOTO and CASTILLA 2004)), revealing the utility of studying yeast prions. In addition, [*PSI*$^{+}$] can be propagated in neuroblastoma cells, suggesting that eukaryotic pathways of prion propagation may be somewhat conserved and better understood by studying their mechanisms in model systems such as yeast (KRAMMER *et al.* 2009).

1.4.3.2 Amyloids in disease

There are several other human diseases, many of which are well-known and well-studied, that display phenotypes associated with amyloid aggregates. However, because these aggregates have not been shown to be infectious, they are not termed as prion diseases. For example, Huntington's disease is characterized by aggregates of a polyglutamine-rich protein, similar to yeast prions, which is able to propagate in a similar fashion (REN *et al.* 2009)). Another common example is the Aβ (Beta-amyloid) peptide, which is implicated in both genetic and spontaneous forms of Alzheimer's disease (reviewed in (HARDY and SELKOE 2002)). Interestingly, this peptide can also be toxic in yeast (TREUSCH *et al.* 2011). A screen was then performed to find genetic factors that modulated Aβ toxicity, and identified multiple human homologues in endocytic trafficking, several of which are known human risk factors for Alzheimer's (TREUSCH *et al.* 2011). Therefore, while these aggregates are not known to be infectious like human or yeast prions, the parallels they share with these proteins illuminate the need to further study the commonalities among them.

1.4.4 ARE PRIONS ADVANTAGEOUS?

Although prions in humans have been described as disease-causing, the yeast prion community is caught in a high degree of controversy over whether or not they are disadvantageous in yeast. The existence of a species barrier, which can prevent transmission of a prion to an outside strain of yeast, as well as the inability to detect many prion variants in the wild, have led some to conclude that yeast prions are a disease (BATEMAN and WICKNER 2012; NAKAYASHIKI *et al.* 2005). In addition, several [*PSI*$^{+}$] variants are lethal, indicating that their presence is merely tolerated in weaker [*PSI*$^{+}$] varieties due to the their mild phenotypes (MCGLINCHEY *et al.* 2011).

Many unfavorable phenotypes have been associated with specific prions, which may also imply a negative impact on growth (TYEDMERS *et al.* 2008). However, prions allow yeast to respond to environmental changes (TYEDMERS *et al.* 2008). This "survival advantage" might be especially relevant with phenotypes related to [*PSI*$^{+}$]-mediated nonsense suppression which could have its advantages to evolution too (TRUE *et al.* 2004). Additionally, recent studies done in wild yeast to measure phenotypes of strains grown in the absence or presence of guanidine hydrochloride to inhibit Hsp104 function suggest that prions do broadly exist in the wild, and can confer selective advantages (HALFMANN *et al.* 2012). It is highly likely that both arguments truthfully represent the broader implications of prions in yeast, which may very well depend on the environment examined. It remains to be seen if these arguments can also be applied to higher organisms.

1.5 THESIS AIMS

In a screen for genes that were required for viability in the absence of the transcription factor *RTF1*, the ubiquitin ligase Rkr1 was uncovered. Other genes from this screen were connected to transcription, suggesting that Rkr1 might also be related to this function. Recent studies have implicated Rkr1 in the quality control of nonstop proteins, produced from translational read-through of stop codons or from translation of nonstop mRNAs which lack a stop codon altogether. However, the basis of lethality between a transcription factor and this ubiquitin ligase is unknown. The foundation of my thesis work is to understand this genetic relationship. I sought to answer several questions to clarify this connection. For example, what are the substrates for Rkr1 and are they related to transcription? With what partners does Rkr1 interact within the cell?

What are the phenotypes associated with loss of *RKR1* in the cell? Are there mechanisms to suppress *rtf1Δ rkr1Δ* lethality that may identify additional functions for these proteins? Using multiple genetic and biochemical techniques, I addressed each of these questions and others to better understand the role of these proteins in *S. cerevisiae.*

A genetic screen to find mutations that rescued lethality of *rtf1Δ rkr1Δ* strains identified three genes that allowed viability in this context. I found that mutations in the genes encoding the heat shock protein, Hsp104, the sister chromatid cohesion protein Chl1, as well as in the RNA Polymerase III subunit Rpc17, suppress *rtf1Δ rkr1Δ* lethality. Further investigations into these results showed that by loss of *HSP104*, cells are alleviated of the prion [*PSI*$^{+}$], which causes increases in nonstop protein and aberrant mRNAs. This and other studies indicated the need for Rtf1 and Rkr1 in the face of [*PSI*$^{+}$]-mediated stress. The basis for obtaining *CHL1* and *RPC17* suppressor mutations is currently unknown; however, these genes have interesting functions in chromatid cohesion and translation. These results allow speculation and planning for exciting and previously unexplored connections between these factors and transcription, chromosome segregation, and translation.

I have performed several experiments and used a variety of approaches to identify substrates or interacting partners for Rkr1. While the results did not uncover any novel interactions, I will discuss in this thesis how my results provide evidence for the quality control role for Rkr1. Additionally, I have uncovered an important phenotype for *rkr1Δ* cells, sensitivity to cycloheximide, which I have used to identify potential interacting partners for Rkr1, as well as important histone residues and proteins that share this phenotype and likely parallel Rkr1 function.

Collectively, my thesis research has provided additional understanding of Rkr1 as a quality control factor that is sensitive to the presence of the prion [*PSI*$^{+}$] and changes in transcription-related processes. These results suggest that the additional stress caused by loss of Rtf1-mediated H2B ubiquitylation is lethal because this function is required to buffer cells against protein and/or mRNA quality control problems in yeast.

2.0 TRANSPOSON MUTAGENESIS TO IDENTIFY SUPPRESSORS OF SYNTHETIC LETHALITY IN YEAST LACKING *RTF1* AND *RKR1*

2.1 INTRODUCTION

Eukaryotic transcription is a highly regulated process which occurs within the controlled and regulated context of chromatin. During transcription, various factors modify chromatin in coordination with elongating RNA polymerase (RNA Pol II) in order to successfully transcribe the DNA template. Changes in chromatin include ATP-dependent chromatin remodeling, the exchange of histone variants, and histone modifications such as methylation, ubiquitylation, and acetylation. The Paf1 complex (Paf1c) is one set of factors that is required for the correct pattern of specific histone modifications (reviewed in (CRISUCCI and ARNDT 2011b; JAEHNING 2010)). This protein complex is conserved from yeast through higher eukaryotes and is present with RNA Pol II on all actively transcribed genes examined, implicating a role for Paf1c in transcription elongation (MAYER *et al.* 2010). Consistent with this, Paf1c in *Saccharomyces cerevisiae* consists of Paf1, Ctr9, Leo1, Cdc73, and Rtf1, and loss of these members causes genetic interactions with mutations in other transcription elongation factor genes and phenotypes associated with defects in elongation (COSTA and ARNDT 2000; MUELLER and JAEHNING 2002; SHI *et al.* 1997; SQUAZZO *et al.* 2002).

Of the histone modifications regulated by members of Paf1c, Rtf1 plays a prominent role in regulating mono-ubiquitylation of K123 on H2B (NG *et al.* 2003a; TOMSON *et al.* 2011; WARNER *et al.* 2007; WOOD *et al.* 2003b). Rad6 is the ubiquitin conjugating enzyme (E2) for this mark, while Bre1 is the ubiquitin ligase (E3) (HWANG *et al.* 2003). This modification is an example of histone crosstalk, as it is a prerequisite for methylation at K4 and K79 of H3 by the Set1 and Dot1 methyltransferases, respectively (DOVER *et al.* 2002; NG *et al.* 2003a; SUN and ALLIS 2002; WOOD *et al.* 2003b). These histone modifications are conserved through humans, where errors in these processes can lead to aberrant gene expression and tumorigenesis. For example, depletion of hBre1 can result in decreased expression of histone genes and the p53 tumor suppressor (SHEMA *et al.* 2008). Conserved patterns of histone modifications are also indicative of transcriptionally active or inactive genes (MINSKY *et al.* 2008).

Paf1c has several roles in addition to its requirement for specific histone modifications, including proper transcript 3'-end formation (MUELLER *et al.* 2004; NAGAIKE *et al.* 2011; NORDICK *et al.* 2008a; PENHEITER *et al.* 2005). The absence of Paf1c members in mammalian cells causes decreased mRNA cleavage, polyadenylation, and mRNA export to the cytoplasm of a VP16 RNA, as well as aberrantly processed and polyadenylated histone mRNAs (FARBER *et al.* 2010; NAGAIKE *et al.* 2011). Human Cdc73 also physically associates with RNA 3' cleavage factors CPSF and CstF (ROZENBLATT-ROSEN *et al.* 2009). In yeast, loss of Paf1c members results in decreased poly(A) tail length and alternative poly(A) site usage (MUELLER *et al.* 2004; STRAWN *et al.* 2009). These studies suggest that the Paf1 complex is essential for both the proper expression and processing of a subset of RNAs and that loss of the Paf1 complex may result in aberrantly formed transcripts that may not be efficiently exported or translated; therefore, these

species could be targeted by mRNA quality control pathways (PENHEITER *et al.* 2005) and (reviewed in (CRISUCCI and ARNDT 2011b; JAEHNING 2010)).

RKR1, which is required for viability in the absence of the Paf1c member Rtf1, encodes a RING finger ubiquitin ligase required for the quality control of nonstop proteins in yeast (BENGTSON and JOAZEIRO 2010; BRAUN *et al.* 2007; WILSON *et al.* 2007). Nonstop proteins are the translational result of nonstop mRNAs, which lack stop codons, or translational read-through of normal stop codons. Both nonstop mRNAs and nonstop proteins are targets for quality control (FRISCHMEYER *et al.* 2002; VAN HOOF *et al.* 2002; WILSON *et al.* 2007). Further, mutations of *RKR1* homologs in higher eukaryotes have been implicated in colon cancer and neurodegeneration, suggesting an important role in cell homeostasis (CHU *et al.* 2009; IVANOV *et al.* 2007). Interestingly, *RKR1* is also required for proper growth of strains with a histone *htb1-K123R* mutation, suggesting that Rkr1 functions in a pathway parallel to the histone modification functions of Rtf1, particularly H2B ubiquitylation, to promote an essential cellular goal (BRAUN *et al.* 2007). However, how this protein quality control factor genetically interacts with Rtf1 is not understood.

To investigate the relationship between Rtf1 and Rkr1, I performed a transposon-mediated mutagenesis screen to identify suppressors of *rtf1Δ rkr1Δ* synthetic lethality in yeast. Through this screen, I confirmed that mutations in genes encoding the chaperone Hsp104, sister chromatid cohesion protein Chl1, and RNA polymerase III subunit Rpc17 rescue lethality of *rtf1Δ rkr1Δ* strains. Further analysis of the mutation in *HSP104* revealed that some *RKR1* genetic interactions occur only in the presence of the prion [*PSI*$^{+}$] (See Chapter 3 for these results), indicating that the presence of this prion negatively influenced strains lacking *RKR1*. In this

chapter, I will discuss further characterization of these three suppressor mutations and their effects on *rtf1Δ* or *rkr1Δ* phenotypes and genetics.

2.2 MATERIALS AND METHODS

2.2.1 Yeast strains and growth conditions

KY *Saccharomyces cerevisiae* strains are isogenic with FY2, a *GAL2*$^{+}$ derivative of S288C (WINSTON *et al.* 1995). Yeast deletion mutants, crosses, and transformants were created using standard protocols (AUSUBEL 1988; ROSE 1991). Yeast were grown on rich (YPD), synthetic complete (SC), synthetic minimal (SD), 5-fluoroorotic acid (5-FOA) or sporulation media as specified and prepared as previously described (ROSE 1991). 6AU plates were made by adding 6-azauracil to SC-URA media to a final concentration of 50 μg/ml.

2.2.2 Yeast dilution assays

Unless otherwise noted, dilution analysis was performed as follows. Specified strains were grown to saturation at 30^{o}C in rich or selective media, washed with sterile water, and diluted into 1x10^{8} cell/ml stocks from which 10-fold serial dilutions were made. Two microliters of cell suspension were plated on appropriate control and selective media plates and were incubated at 30^{o}C for the specified number of days (2 to 7).

2.2.3 Plasmids

The *his3* nonstop plasmid, pAV240 (*LEU2*), and protein A nonstop plasmid, pAV184 (*URA3*), were gifts from Dr. Ambro van Hoof (WILSON *et al.* 2007). The sup35NM-GFP (*URA3* or *LEU2*) plasmids were gifts from Dr. Susan Liebman (ZHOU *et al.* 2001). The *URA3*-marked plasmid carrying *RTF1*, pKA69, was used to maintain *rtf1Δ rkr1Δ* viability (STOLINSKI *et al.* 1997). 2µ *RTF1* (pAP45), empty vector (pAP37), Rtf1 HMD (residues 66-152) (pAP39) or Rtf1 HMD-E104K (pAP54) *TRP1*-marked plasmids were constructed by cloning the indicated gene under control of an *ADH1* promoter and N-terminal Myc-tag. For the HMD and HMD-E104K constructs, the Large T antigen nuclear localization signal was included before the Myc tag (Piro et al, PNAS, in press). The *ade1-14* allele was amplified from a strain from Dr. Susan Liebman and cloned using XmaI and SacI sites into pRS306 for integration into our strain background via a two-step integration (BRADLEY *et al.* 2003).

2.2.4 Mutagenesis and confirmation of genetic suppressors

Transposon mutagenesis to identify suppressors of *rtf1Δ rkr1Δ* synthetic lethality was performed by transforming a *LEU2*-marked set of integrating plasmids (described in (KUMAR *et al.* 2000)) into an *rtf1Δ rkr1Δ* strain (KY1663) carrying *RTF1*/*URA3/CEN/ARS* (pKA69) and selecting on SC-LEU medium. Transformants were replica-plated onto SC-LEU medium containing 5-FOA to select for colonies that had lost the *RTF1/URA3* plasmid. Fifteen-thousand colonies were screened and fifty-five candidates were purified and analyzed further. Approximately 30,000 transformants would be necessary to cover 98% of the yeast genome (described in (KUMAR *et al.* 2000)). Stable integration of the transposon was verified by streaking strains onto YPD and

replica-plating onto SC-LEU medium containing 5-FOA. Thirty-nine candidates passed this test and were taken from 5-FOA plates for further analysis. These strains, *rtf1Δ rkr1Δ TnSup::LEU2,* were then used in backcrosses with an *rtf1Δ* (KMKY139) strain containing pKA69, as a functional copy of *RTF1* is required for sporulation (data not shown). Tetrad analysis of these crosses confirmed that only one Tn was present per candidate by 2:2 sorting of *LEU2*. This cross also confirmed that every *rtf1Δ rkr1Δ* Leu^- spore was $5\text{-}FOA^S$ while every *rtf1Δ rkr1Δ* Leu^+ spore was $5\text{-}FOA^R$, verifying linkage of the Tn to suppression of lethality. Fifteen *rkr1Δ TnSup::LEU2* strains exhibited 2:2 sorting of the transposon, and the transposon was linked to suppression of lethality. These fifteen candidates were taken through a second backcross with an *rtf1Δ* strain (KY619) to verify that the transposon rescued *rtf1Δ rkr1Δ* lethality independently of pKA69 and 5-FOA. In this case, we expected suppression of lethality only in *rtf1Δ rkr1Δ TnSup::LEU2* strains. Three Tn mutants passed these genetic criteria, and the Tn insertion was recovered as previously described by rescuing the insertion in yeast with linearized pRSQ2-*URA3* and the plasmid insert was sequenced with an M13 oligo (BURNS *et al.* 1994). The Tn candidate designated 5-5 had a Tn insertion located 70 base pairs (bp) 3' to the stop codon of *RPC17*. The second candidate, 13-5, mapped within *HSP104*, 68 bp from the end of the open reading frame. The third, 13-7, contained a Tn insertion 43 bp into the open reading frame of *CHL1*. Mutations in all three genes were verified to suppress *rtf1Δ rkr1Δ* lethality by creating clean KanMX replacements of *CHL1* or *HSP104*, or by the use of the *rpc17*-DAmP allele (Open Biosystems) and performing tetrad analysis of a triply heterozygous diploid (*rtf1/RTF1 rkr1Δ/RKR1 sup/SUP*).

2.2.5 Live-cell imaging

Strains were transformed with a Sup35NM-GFP (ZHOU *et al.* 2001) plasmid to test for the presence of [*PSI*$^{+}$] and patched onto selective media containing 100 µM $CuSO_4$. Plates were protected from light and incubated at 30^{o}C for several days to ensure actively growing cells before live cell imaging. Imaging was performed on wet mounts using a Leica TCS SP5 Confocal Microscope (Leica Microsystems, Buffalo Grove, IL). Observations were made of at least three transformants per strain and hundreds of cells per transformant. Representative images are shown for Sup35NM-GFP fluorescence.

2.2.6 Western analysis

For Protein A nonstop western analysis, strains transformed with pAV184 (WILSON *et al.* 2007) were grown at 30^{o}C in SC-Ura liquid medium containing 2% galactose to an OD_{600} of 0.7 to 0.9. Cells were normalized to 10.8 OD_{600} units. For histone methylation levels, strains were grown in YPD to 2.66 OD_{600} units. Extracts were made using glass bead lysis in 20% trichloroacetic acid as previously described (COX *et al.* 1997; ZHENG *et al.* 2010). An equal amount of each extract (5 µl) was run on a 15% SDS polyacrylamide gel and transferred to nitrocellulose membrane for western analysis using standard methods. Briefly, the membrane was probed with Peroxidase-Anti-Peroxidase (1:2000 dilution; Sigma) to assay levels of Protein A and anti-G6PDH antibody (1:50,000; Sigma) as a loading control for the Protein A blots. For the histone methylation levels, membranes were probed with anti-H3 K4 me2 (1:2,000, Millipore) or anti-H3 (1:50,000 Custom made by GenScript). Immunoreactivity was measured using chemiluminescence (Perkin-Elmer) and a 440 CF digital imaging station (Kodak).

2.3 RESULTS

2.3.1 *rtf1Δ rkr1Δ* synthetic lethality is rescued by restoring Rtf1-dependent histone modifications

Patrick Costa and Mary Braun previously identified *RKR1* in a screen for mutations that cause synthetic lethality in the absence of *RTF1* and showed that mutations in several genes required for H2B ubiquitylation, including *HTB1*, *RAD6*, and *BRE1*, cause synthetic growth defects in combination with *rkr1*Δ (BRAUN *et al.* 2007). These results suggested that the H2B ubiquitylation function of Rtf1 is required for normal cell growth in the absence of Rkr1. To test this idea further, I asked whether a 90-amino acid histone modification domain (HMD)-containing fragment of Rtf1 could complement an *rtf1*Δ mutation and restore viability to an *rkr1*Δ *rtf1*Δ double mutant. Anthony Piro recently demonstrated that this Rtf1 HMD fragment, which includes amino acids 62-152 of Rtf1, is sufficient for promoting H2B ubiquitylation and downstream methylation of H3 in the absence of all other parts of the Rtf1 protein (*Piro et al*, PNAS, In press). Using a plasmid shuffle assay, I found that expression of either full-length Rtf1 or the isolated HMD fragment rescued lethality of an *rtf1Δ rkr1Δ* strain (Figure 7). In contrast, a mutant version of the HMD, HMD-E104K, which is defective in promoting Rtf1-dependent histone modifications (*Piro et al*. PNAS, In Press) (TOMSON *et al.* 2011) did not rescue *rtf1Δ rkr1Δ* synthetic lethality, even though it is expressed at similar levels as the wild-type HMD. Together with our previous genetic data, these results strongly suggest that, with respect to *RTF1*, a primary cause of the synthetic lethality between *rtf1*Δ and *rkr1*Δ is the absence of H2B K123 ubiquitylation and its downstream effects.

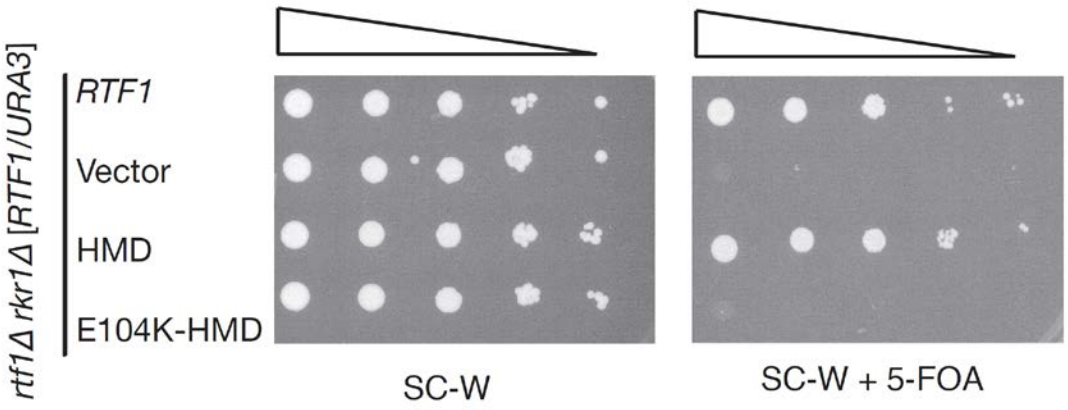

Figure 7: Restoration of histone modifications rescues *rtf1Δ rkr1Δ* synthetic lethality

2μ *TRP1*-marked plasmids expressing the indicated form of *RTF1* or empty vector were transformed into an *rtf1Δ rkr1Δ [RTF1/URA3]* strain (KY2205) and dilution analysis was performed on SC-W or SC-W 5-FOA. Plates were incubated at 30^{o}C for 3 days.

2.3.2 Identification and verification of Tn mutations that suppress *rtf1Δ rkr1Δ* synthetic lethality

To further investigate the basis of lethality in strains deficient in H2B ubiquitylation through loss of Rtf1 and the ubiquitin ligase Rkr1, I used a transposon-based (Tn) mutagenesis strategy to

find suppressors of the *rtf1Δ rkr1Δ* synthetic lethal interaction (KUMAR *et al.* 2000). A library of plasmids containing yeast genomic DNA and *LEU2*-marked transposon insertions was transformed into an *rtf1*Δ *rkr1*Δ strain, which carried a *URA3*-marked *RTF1* plasmid for viability. Approximately 15,000 transformants were screened for loss of the *URA3*-marked *RTF1* plasmid on medium containing 5-FOA. Following phenotypic confirmation, candidates were crossed to an *rtf1Δ* strain containing an *URA3*-marked *RTF1* plasmid to verify 2:2 sorting of the *LEU2*-marked transposon, indicating only one insertion site, as well as linkage of the 5-FOA resistance to the *LEU2* marker in *rtf1*Δ *rkr1*Δ colonies. Fifteen strains met these requirements, and *rkr1Δ Tnsup::LEU2* strains from those crosses were backcrossed to an *rtf1Δ* strain to verify that the Tn insertion rescued lethality of the *rtf1*Δ *rkr1*Δ double mutants in the absence of a plasmid source of *RTF1*. Three candidate strains met these criteria and were chosen for further analysis. For the other 12 candidates, spontaneous *rtf1*Δ *rkr1*Δ spores arose during this backcross, and therefore were not further analyzed at this time due to suspicion of other suppressors in the cross. (This is further discussed in the conclusions of this thesis.)

The transposon insertions were recovered and sequenced in the three final candidates by plasmid rescue (BURNS *et al.* 1994), revealing transposon insertions in the 3' coding region of *HSP104*, the 5' coding region of *CHL1*, and the 3'-UTR of *RPC17*. *HSP104* encodes a heat shock protein involved in the maintenance and propagation of aggregated and misfolded proteins, including yeast prions (reviewed in (GRIMMINGER-MARQUARDT and LASHUEL 2010)). Chl1 is a putative DNA helicase required for sister chromatid cohesion, as well as proper transcriptional silencing of the *HMR* loci, and DNA damage repair (DAS and SINHA 2005; LAHA *et al.* 2006; XU *et al.* 2007). Rpc17 is an essential and specific subunit of RNA polymerase III

(Pol III) that physically interacts with TFIIIB70, Rpc11, and Rpc31, likely indicating a role for this protein in transcription initiation of Pol III transcribed genes (FERRI *et al.* 2000).

To confirm that mutations in the three suppressor genes rescued *rtf1*Δ *rkr1*Δ synthetic lethality, the *HSP104* and *CHL1* genes were deleted and replaced by a KanMX cassette and the resulting strains were crossed to a *rkr1Δ* strain. For *RPC17*, which encodes an essential gene, an *rpc17-DAmP* allele with a mutation also within the 3'UTR was obtained (Open Biosystems) and crossed to a *rkr1Δ* strain. The DAmP (*D*ecreased *A*bundance by *m*RNA *P*erturbation) mutation contains a KanMX cassette inserted immediately following the open reading frame and likely causes decreased *RPC17* transcript levels (not verified) (BRESLOW *et al.* 2008). Double mutants from these three crosses were then mated with an *rtf1Δ* strain. The diploids, which are heterozygous for three genes (*RTF1*, *RKR1*, and *HSP104*, *CHL1*, or *RPC17*), were subjected to tetrad analysis. As expected, double mutant spores lacking *RTF1* and *RKR1* were inviable or very slow growing in the crosses with a *rpc17-DAmP* or a *chl1Δ* mutation. Mutations in *RPC17* or *CHL1* suppressed this lethality in all cases (Figure 8). Surprisingly, spores lacking *RTF1* and *RKR1* were alive and healthy in crosses where the diploid was heterozygous for *HSP104* (This result is further examined in Chapter 3). Confirming the identification of *HSP104* in our suppressor screen, *hsp104Δ rtf1Δ rkr1Δ* triple mutants were also alive (Figure 8).

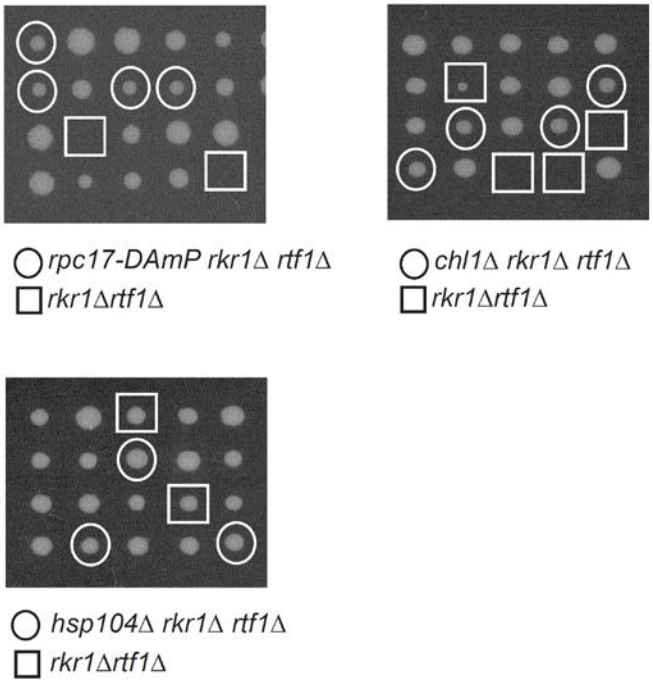

Figure 8: Mutations in *HSP104, CHL1*, and *RPC17* rescue *rtf1Δ rkr1Δ* synthetic lethality

Shown are tetrad dissections of crosses between *rkr1Δ rpc17-DAmP, rkr1Δ chl1Δ,* or *rkr1Δ hsp104Δ* strains and an *rtf1Δ* strain (KY958). Dissections were done on YPD and incubated at 30^{o}C for 3 days**.** All spores have been assigned to ensure proper sorting of the tetrad. For simplicity, double *rtf1Δ rkr1Δ* mutants are highlighted by boxes while triple mutants are highlighted by circles.

2.3.3 Mutations in *HSP104, CHL1,* and *RPC17* and suppression of *rtf1Δ* or *rkr1Δ* phenotypes

To better understand the mechanism of suppression, I tested which phenotypes of *rtf1Δ* or *rkr1Δ* cells were rescued by these suppressor mutations. These results would indicate whether mutations in *HSP104*, *CHL1*, or *RPC17* were alleviating the loss of *RTF1* or *RKR1* and therefore help to elucidate the role of the factor in suppression of *rtf1Δ rkr1Δ* lethality.

2.3.3.1 Suppression of *rkr1Δ* genetic interactions

In addition to synthetic lethality with *rtf1Δ* and H2B K123R mutations, loss of *RKR1* is also synthetically lethal with loss of *SPT10*, which is required for proper histone gene expression (BRAUN *et al.* 2007; DOLLARD *et al.* 1994). Therefore, to investigate if mutations in *CHL1*, *HSP104*, or *RPC17* could also rescue this genetic interaction, I performed tetrad analysis of a *rkr1Δ*/*RKR1 spt10Δ*/*SPT10 sup*/*SUP* (*HSP104*, *RPC17*, or *CHL1*) strain and found that only deletion of *hsp104Δ* could rescue *rtf1Δ spt10Δ* synthetic lethality (Table 1). This result indicated that loss of *HSP104* likely rescued a *rkr1Δ*-specific defect.

Table 1: Effect of suppressors on *rtf1Δ* or *rkr1Δ* genetic interactions

Double Mutant	Genetic interaction	Suppression upon introduction of *chl1Δ*, *hsp104Δ*, or *rpc17-DAmP*
rtf1Δ rkr1Δ	Lethal	Viable with *chl1Δ*, *hsp104Δ*, and rpc17-DAmP
rtf1Δ swi4Δ	Lethal	No suppression
rtf1Δ htz1Δ	Very sick	Very slight with *hsp104Δ*
rtf1Δ arg82Δ	Very sick	Very slight with *hsp104Δ*
rtf1Δ spt4Δ	Very sick	No suppression
rkr1Δ spt10Δ	Lethal	Suppression with *hsp104Δ* to *spt10Δ*-level growth

2.3.3.2 Suppression of *rtf1Δ* genetic interactions

Loss of *rtf1Δ* causes sickness or lethality in combination with several mutations, including *swi4Δ*, *htz1Δ*, *arg82Δ*, and *spt4Δ* (Costa and Arndt, unpublished results and (COSTA and ARNDT 2000; PORTER *et al.* 2002)). To investigate if any of these genetic interactions with *rtf1Δ* were rescued by loss of *CHL1*, *HSP104*, or *RPC17*, I performed tetrad analysis of diploids heterozygous for three genes. Interestingly, I did not observe suppression of the *rtf1Δ* genetic interactions in these crosses (Table 1), other than a very slight increase in growth of *htz1Δ rtf1Δ hsp104Δ* triple mutants compared to *htz1Δ rtf1Δ* double mutants or *arg82Δ rtf1Δ hsp104Δ* strains compared to *arg82Δ rtf1Δ* double mutants (Table 1). However, this was rather weak suppression of the *htz1Δ rtf1Δ* and *arg82Δ rtf1Δ* growth defects. Also, these strains frequently picked up

suppressor mutations that enhanced growth (personal observation, data not shown), which made further analysis difficult. As will be further discussed in Chapter 3, the loss of *HSP104* clears cells of [*PSI*$^{+}$] and alleviates a stress on cells lacking *RKR1*, which is involved in controlling levels of nonstop proteins. Interestingly, both *ARG82*-related processes and *HTZ1* have also been shown to play a role in nonstop quality control (WILSON *et al.* 2007). Therefore a small part, but not all, of the genetic interaction between *rtf1Δ* and these genes may be due to the nonstop quality control pathway.

2.3.3.3 Suppression of *rtf1Δ* growth phenotypes

Loss of *RTF1* causes phenotypes usually associated with defects in transcription. These include a suppressor of Ty (Spt^{-}) phenotype using a *his4-912δ* allele of *HIS4*, which contains a Ty δ (long terminal repeat) element upstream of the TATA box. The δ insertion within the promoter of *HIS4* interrupts wild type growth on medium lacking histidine. Strains with an Spt^{-} phenotype, such as *rtf1Δ* strains, are able to grow on medium lacking histidine by suppressing this promoter defect and altering start-site selection (ROEDER and FINK 1982; STOLINSKI *et al.* 1997). In addition, *rtf1Δ* strains are sensitive to the base analog 6-azauracil (6AU), which causes a reduction in intracellular nucleotide pools and subsequent polymerase stalling, requiring functional elongation factors for continued transcription (EXINGER and LACROUTE 1992; SQUAZZO *et al.* 2002). Therefore, strains with defects in transcription elongation are sensitive to medium containing this compound. To investigate if loss of *CHL1*, *HSP104*, or *RPC17* was able to suppress these *rtf1Δ* defects in transcription, I assessed the growth of *rtf1Δ sup* double mutants on medium lacking histidine or containing 6AU. Although no double mutant suppressed an *rtf1Δ* Spt^{-} phenotype, deletion of *HSP104* or mutation in *RPC17* slightly rescued an *rtf1Δ* 6AU sensitivity phenotype (Figure 9). These results suggest that loss of *HSP104* or *RPC17* may

partially suppress *rtf1Δ*-dependent defects in transcription. Notably, *rpc17-DAmP* strains exhibit slower growth than wild type strains (apparent on earlier days, data not shown), suggesting that suppression of an *rtf1Δ* defect in transcription suppression may be greater than what can be appreciated by this assay.

Rtf1 is required for the regulation of H2B K123 ubiquitylation and downstream methylation on H3 K4 and H3 K79 by Set2 and Dot1, respectively (NG *et al.* 2003a; TOMSON *et al.* 2011; WARNER *et al.* 2007; WOOD *et al.* 2003b). To assess if the identified suppressor mutations were able to restore Rtf1-dependent histone modifications, I performed a western analysis of H3 K4 di-methylation levels in double mutant strains. However, none of these strains had detectable levels of this mark (Figure 10). These data suggest that defects in Rtf1-dependent histone modifications were not suppressed in these strains and this was not the reason for restoring viability to *rtf1Δ rkr1Δ* double mutants.

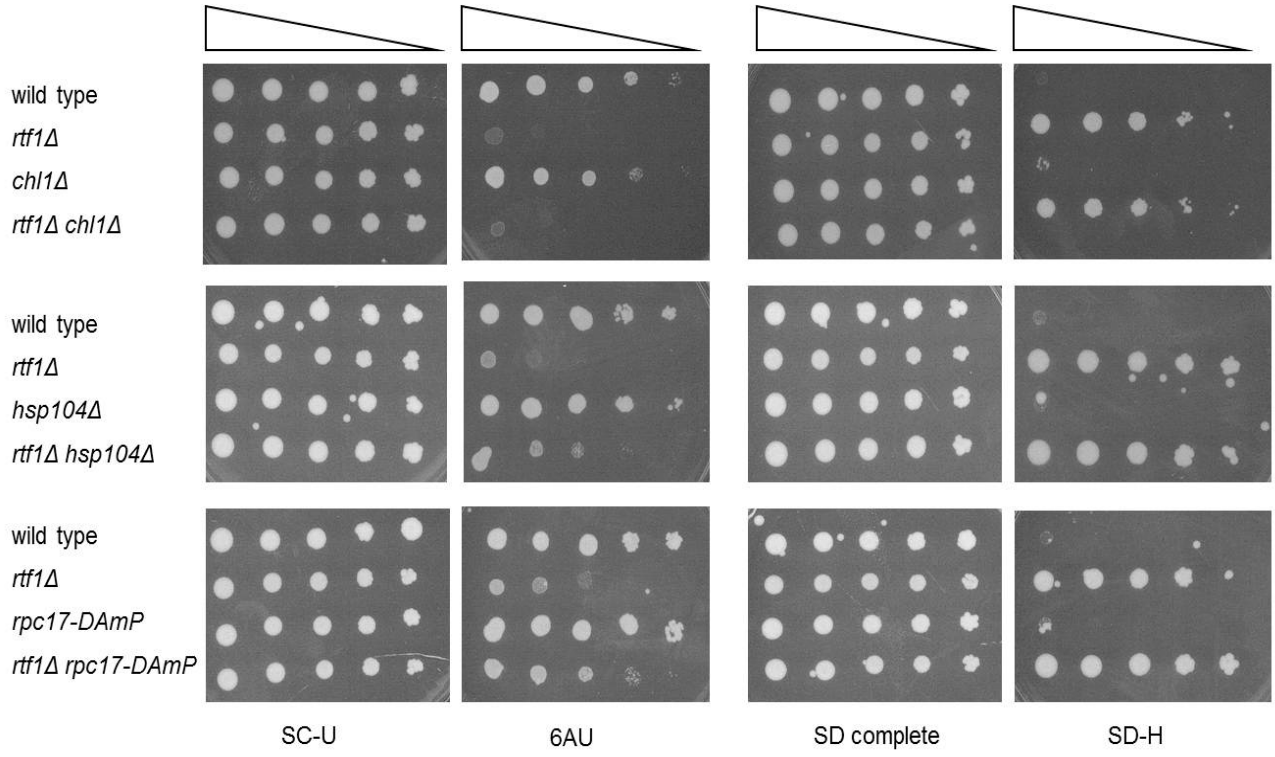

Figure 9: Effects of suppressor mutations on *rtf1Δ* spt$^-$ or 6AU^S phenotypes

Wild type (KY1008), *rtf1Δ* (KY2161), *chl1Δ* (KY2163), *chl1Δ rtf1Δ* (KY2162), *hsp104Δ* (KY2165), *hsp104Δ rtf1Δ* (KY2164), *rpc17-DAmP* (OKA249), *rtf1Δ rpc17-DAmP* (OKA248) and strains were used for dilution growth assays on SC-U, SC-U+6AU, SD Complete or SD-H and incubated at 30^{o}C for 2 to 4 days.

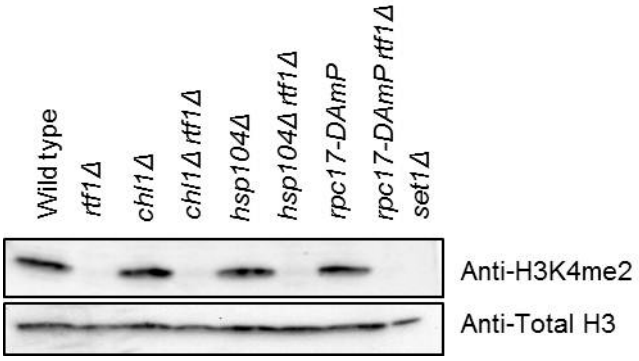

Figure 10: Mutation of *HSP104*, *CHL1*, or *RPC17* does not rescue an *rtf1Δ* defect in H3 K4 di-methylation

Wild type (KY1019), *rtf1Δ* (KY2161), *chl1Δ* (KY2162), *chl1Δ rtf1Δ* (KY2162), *hsp104Δ* (KY2165), *hsp104Δ rtf1Δ* (KY 2164), *rpc17-DAmP* (OKA249), *rpc17-DAmP rtf1Δ* (OKA248), and *set1Δ* strains were grown to log phase in YPD. Extracts were made using TCA and an equal amount of extract was loaded onto two separate 15% SDS POLYACRYLAMIDE gels. The gels were transferred to nitrocellulose and probed with anti-H3 K4me2 or anti-H3 as a loading control.

2.3.4 Genetic interactions of *rtf1Δ* and *rkr1Δ* mutations with defects in mRNA degradation pathway components

Loss of *RKR1* causes an increase in nonstop protein levels (BENGTSON and JOAZEIRO 2010; WILSON *et al.* 2007). Rtf1 is a member of the Paf1c, which is required for proper mRNA formation (reviewed in (CRISUCCI and ARNDT 2011b; JAEHNING 2010)). Therefore, loss of *RTF1*

may increase levels of aberrant mRNA. I hypothesized that combined defects in protein and mRNA quality control from the loss of *RKR1* and *RTF1*, respectively, may be lethal to yeast. To test this hypothesis, I obtained strains with mutations in genes with known roles in mRNA quality control (*SKI7*, *SKI3*, *XRN1*, *UPF1*, *UPF3*, *TRF4*, and *RRP6*). If loss of *RTF1* increases levels of abnormal RNA, then deletion of genes encoding mRNA quality control pathway members, which are known to increase aberrant transcripts, may also be synthetically lethal with *RKR1*. These genes represent several mRNA quality control pathways, including the nuclear and cytoplasmic exosome, nonsense-mediated decay, and nonstop mRNA degradation (reviewed in (FASKEN and CORBETT 2005)).

I performed a tetrad analysis of heterozygous double mutant diploids mutated in *RKR1* and one of the above quality control genes. Interestingly, I only found that the deletion of *XRN1* causes extreme sickness or lethality in combination with *rkr1Δ* (data not shown). This original cross was performed using an *xrn1Δ* strain from the yeast deletion collection, and so I verified this genetic interaction by creating an *XRN1* deletion in our strain background and repeating this tetrad analysis (Figure 11). *XRN1* encodes a cytoplasmic 5'→ 3' exonuclease which decaps and degrades aberrant transcripts, including those targeted for nonsense mediated decay (reviewed in (FASKEN and CORBETT 2005)). Although these data do not prove that loss of *RTF1* increases substrates for Xrn1-mediated decay, it does suggest that strains lacking *RKR1* are indeed sensitive to defects in mRNA quality control and increases in aberrant transcripts such as nonsense mRNAs or improperly processed mRNAs. The connection between these two pathways remains an avenue to be further investigated.

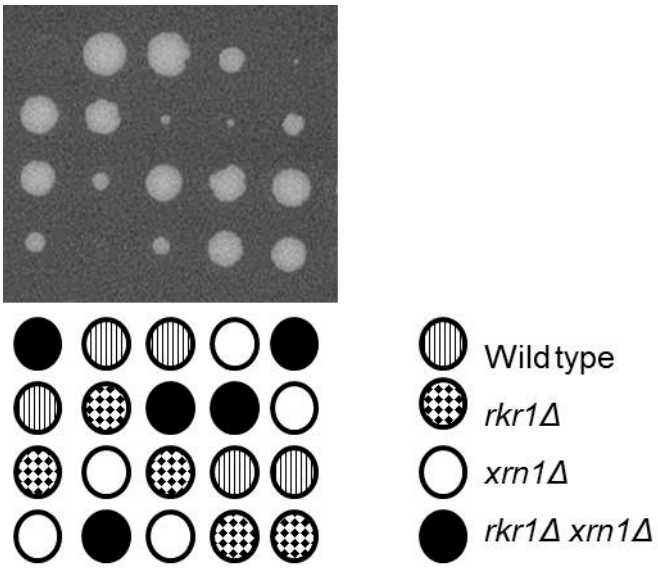

Figure 11: Loss of *RKR1* genetically interacts with loss of mRNA degradation factor *XRN1*

A *rkr1Δ/RKR1* heterozygous diploid (SHY13 crossed by KY1030) was transformed with a PCR product to delete the *XRN1* gene and replace it with a KanMX cassette. Shown is tetrad analysis of one resulting diploid dissected onto YPD and incubated 3 days at 30^{o}C.

2.3.5 Further analysis into suppression of *rtf1Δ rkr1Δ* strains by an *rpc17* or *chl1* mutation

I will show in Chapter 3 that deletion of *HSP104* rescues *rtf1Δ rkr1Δ* synthetic lethality by clearing cells of the prion [*PSI*$^{+}$]. The presence of [*PSI*$^{+}$], an aggregated form of the translation termination factor Sup35 required to efficiently recognize stop codons, causes an increase in nonsense suppression and increased aberrant mRNAs and proteins (TRUE *et al.* 2004; WILSON *et al.* 2005). Clearing cells of [*PSI*$^{+}$] by mutation of *HSP104* relieves these cells of an additional burden quality control pathway. Therefore, in these unpublished investigations, I also analyzed the effects of *rpc17* and *chl1* suppressor mutations on these [*PSI*$^{+}$]-related phenotypes. *RPC17* encodes a member of the RNA Pol III machinery, which transcribes non-messenger RNAs such as tRNAs and 5S rRNA, while *CHL1* encodes a putative DNA helicase implicated in sister chromatid cohesion and transcriptional silencing (DAS and SINHA 2005; FERRI *et al.* 2000; LAHA *et al.* 2006; XU *et al.* 2007).

2.3.5.1 [*PSI*$^{+}$] phenotype

The suppressor mutations that rescue *rtf1Δ rkr1Δ* synthetic lethality represent three proteins associated with very different cellular functions. Of Chl1, Rpc17, and Hsp104, Hsp104 is the best studied. Loss of Hsp104 or mutation of its ATPase domain disrupts the ability of yeast cells to propagate prions, particularly [*PSI*$^{+}$], an aggregate of the translation termination factor Sup35 (CHERNOFF *et al.* 1995; MACKAY *et al.* 2008). To assay if the transposon insertion in *HSP104* identified in my suppressor screen cleared cells of [*PSI*$^{+}$] in our strain background, I transformed this strain and the original strain used in my transposon mutagenesis screen with a GFP-tagged prion domain of Sup35. In [*PSI*$^{+}$] strains, cells exhibit puncta representative of Sup35-GFP

aggregates. The fluorescence is diffuse in cells cleared of [*PSI*$^{+}$] (ZHOU *et al.* 2001). Transformants were analyzed using live-cell imaging with a confocal microscope. Indeed, the transposon insertion in *HSP104* cleared cells of [*PSI*$^{+}$] from our strain background (Figure 12; Further elaborated in Chapter 3). However, the same analysis on the transposon mutations within *CHL1* or *RPC17* showed that these strains still contained [*PSI*$^{+}$], suggesting that suppression by the *CHL1* or *RPC17* mutations is likely occurring through a different mechanism.

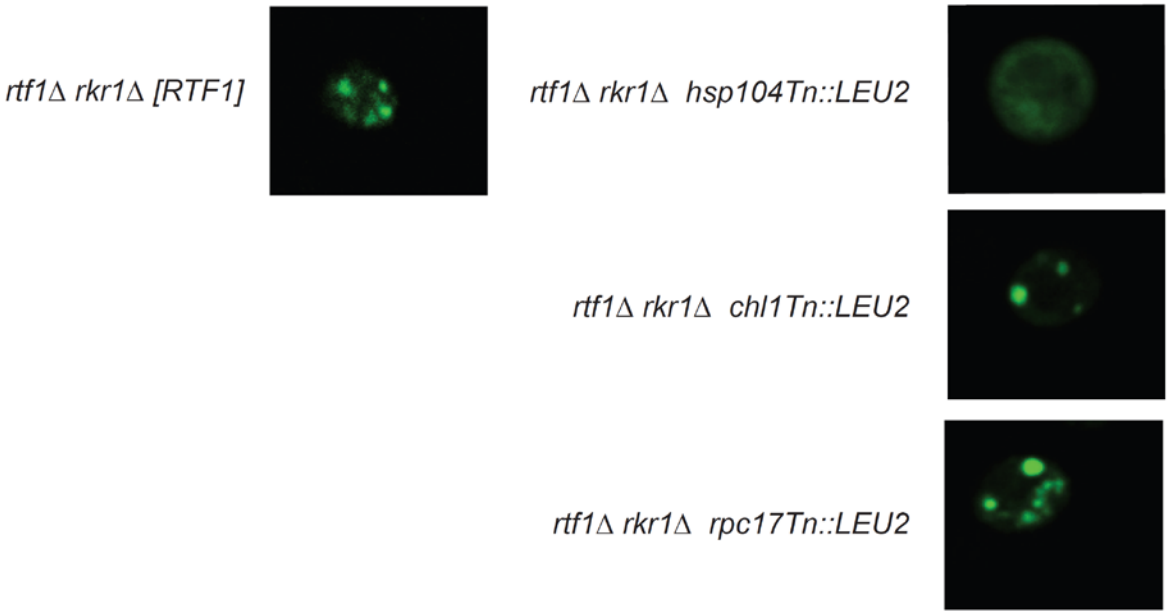

Figure 12: Tn mutations within *HSP104*, but not *CHL1* or *RPC17*, clear cells of [*PSI*$^+$]

The strain originally used for transposon-mediated mutagenesis, an *rtf1Δ rkr1Δ* strain (KY1663) carrying a *URA3*-marked *RTF1* plasmid, was also transformed with a *LEU2*-marked *pCUP1-SUP35NM-GFP* plasmid. The transposon-mutant strains recovered from this screen, *hsp104Tn::LEU2*, *chl1Tn::LEU2*, and *rpc17Tn::LEU2* were transformed with a *URA3*-marked *pCUP1-SUP35NM-* GFP plasmid. Strains were patched onto SC-Leu or SC-Ura plates containing 100 µM $CuSO_4$ and incubated in the dark at 30^{o}C before life-cell imaging use a confocal microscope.

2.3.5.2 Assays to measure nonstop protein levels

Inactivation of *HSP104* rescues lethality between *rkr1Δ* and *rtf1Δ* by curing cells of [*PSI*$^+$]. Most likely, this alleviates cells of accumulated nonstop proteins, which could be targets for Rkr1-dependent quality control. However, it is unclear how *CHL1* and *RPC17* mutations rescue this synthetic lethality since mutations in these genes do not cure cells of [*PSI*$^+$].

I hypothesized that loss of *RTF1* caused an increase in aberrant transcripts lacking a stop codon, and a consequent increase in nonstop protein. This, in conjunction with the increase in nonstop proteins from loss of *RKR1* and the presence of [*PSI*$^+$], lead to synthetic lethality in yeast. To address this question, I used two nonstop protein reporter plasmids. The first contained the *HIS3* gene without a stop codon. Wild type cells carrying the reporter efficiently degrade the *his3* nonstop transcript and protein and cannot grow on media lacking histidine, while cells lacking *RKR1* are unable to degrade the His3 nonstop protein and therefore grow in these conditions (WILSON *et al.* 2007). The second nonstop reporter contained a Protein A gene also lacking a stop codon, and the levels of the resulting protein were assayed by western analysis (WILSON *et al.* 2007). Using these reporters, I found that while loss of *RKR1* increases levels of nonstop reporter proteins, there is a surprising decrease in nonstop protein levels in *rtf1Δ* cells (Figure 13; See also Chapter 3) (WILSON *et al.* 2007).

Although this result could not simply explain synthetic lethality between *rtf1Δ* and *rkr1Δ*, the decrease in nonstop proteins in *rtf1Δ* cells was still an intriguing finding. This indicated that *RTF1* is necessary for the correct levels, processing, mRNA export/stability, or translation of these transcripts/proteins and provided me an additional phenotype with which to further explore the role of my genetic suppressors. Therefore, to identify if mutations in *RPC17*

or *CHL1* could suppress this *rtf1Δ* defect, I assayed the levels of nonstop proteins in these strains.

rpc17-DAmP strains have a slow-growth phenotype, making dilution analyses difficult to interpret. Therefore, I used western analysis to examine the levels of protein A nonstop levels. Although I confirmed that loss of *RKR1* increased nonstop levels and loss of *RTF1* has the opposite effect, I did not observe an effect of *rpc17-DAmP* on total protein A (Figure 13).

I next focused on a potential role in nonstop protein synthesis or stability for *CHL1*. Using both the *his3* and Protein A nonstop reporters, I found that deletion of *CHL1* in a *rkr1Δ* resulted in increased nonstop protein levels compared to a *rkr1Δ* strain alone (Figure 14A). Furthermore, the deletion of *RTF1* suppressed the high nonstop protein levels of *chl1*Δ *rkr1*Δ cells back to wild type levels (Figure 14B).

2.3.5.3 Nonsense suppression

The above results suggest a role for Rtf1 in controlling nonstop protein levels. The Paf1c has also been implicated in altering levels of nonsense transcripts, including decreasing protein levels from an *ade1-14* allele containing a premature stop codon (STRAWN *et al.* 2009). To investigate if a *chl1*Δ mutation could suppress an *rtf1*Δ defect in aberrant transcripts other than nonstop, I performed a dilution analysis on medium lacking adenine with strains containing the *ade1-14* allele in the [*PSI*$^{+}$] background, which allows read-through of the nonsense allele and production of functional Ade1 protein. As previously observed, *rtf1*Δ *ade1-14* strains are unable to grow in this condition (Figure 14C) (STRAWN *et al.* 2009). However, I observed that a *chl1*Δ *rtf1*Δ strain partially suppressed this defect (Figure 14C). Further, a *chl1*Δ mutant exhibited better growth on medium lacking adenine than a wild type strain (Figure 14C).Therefore, consistent with the results of my genetic suppressor screen, I have shown that *rtf1*Δ and *chl1*Δ

act in opposition to one another, by affecting the levels of nonstop and nonsense proteins independent of the presence of prions.

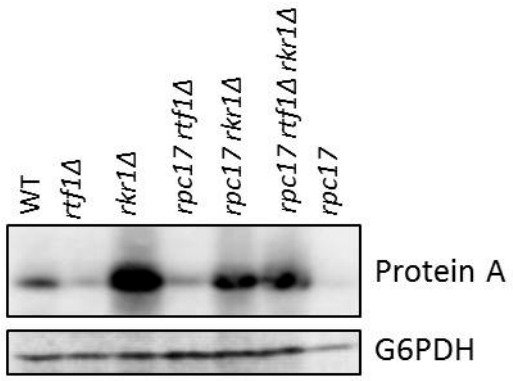

Figure 13: A mutation in the *RPC17* 3' UTR has little effect on nonstop protein levels

WT (KY307), *rtf1Δ(KY2211*), *rkr1Δ* (KY2236), *rpc17-DAmP rtf1Δ* (KMKY178), *rpc17-DAmP rkr1Δ* (KMKY174), *rpc17-DAmP* (KA137), *rpc17-DAmP rkr1Δ rtf1Δ* (KMKY181) strains were transformed with a *URA3*-marked plasmid containing a protein A nonstop reporter and grown to early log phase in SC-U containing 2% galactose to induce expression of the protein A gene. TCA extracts were made and analyzed by western analysis using antibodies against Protein A or G6PDH as a loading control.

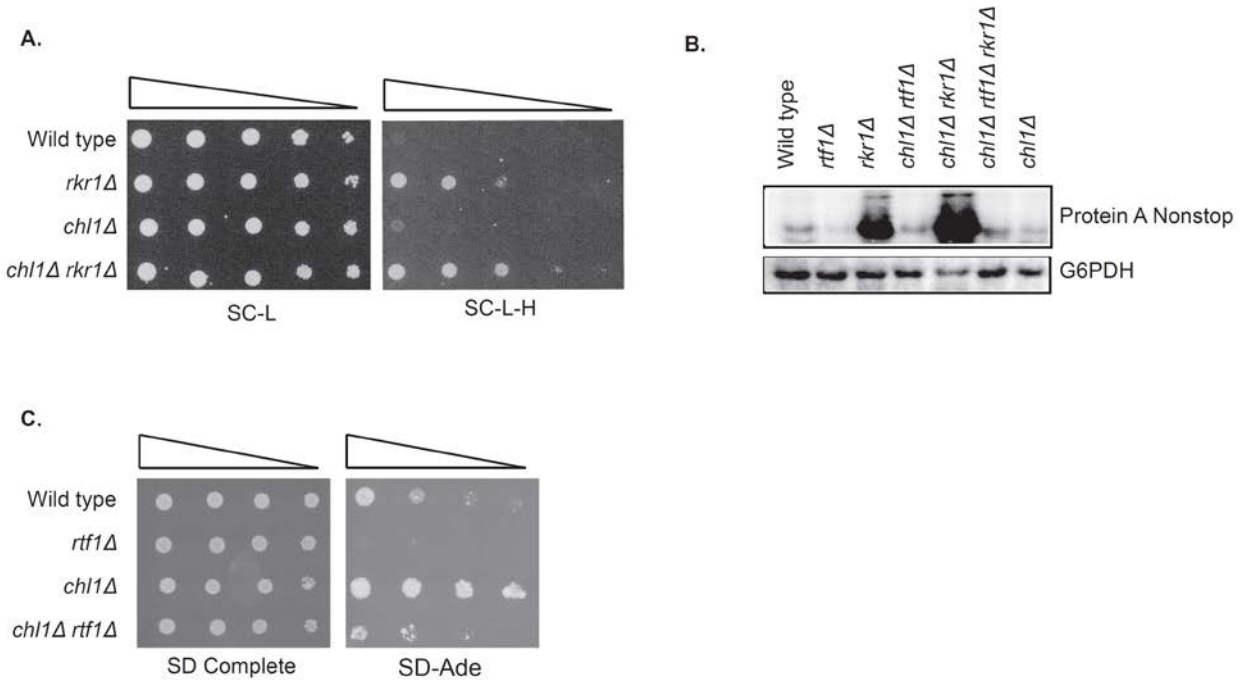

Figure 14: *chl1Δ* suppresses *rtf1Δ* effects on nonsense and nonstop protein levels

(A) WT (KY1030), *rkr1Δ* (KY2236), *chl1Δ* (KY1899) and *chl1Δ rkr1Δ* (KY2210) strains were transformed with a *LEU2*-marked plasmid containing a *his3* non-stop reporter. Ten-fold serial dilutions were performed on SC-Leu or SC-Leu-His plates and incubated at 30°C for 2 days. (B) WT (KY307), *rtf1Δ* (KY2211), *rkr1Δ (*KY2201), *chl1Δ* (KY1898) and *chl1Δ rkr1Δ* (KY2208), *chl1Δ rtf1Δ* (KY2207), *chl1Δ rtf1Δ rkr1Δ* (KY2206) strains were transformed with a *URA3*-marked plasmid containing a protein A nonstop reporter and grown to early log phase in SC-Ura containing 2% galactose to induce expression of protein A. TCA extracts were made and analyzed by western analysis using antibodies against Protein A or G6PDH as a loading control. (C) WT (KY2212), *rtf1Δ* (KY2213) *chl1Δ* (KY2214) *rtf1Δ chl1Δ* (KY2215) strains carrying the *ade1-14* nonsense allele were grown to saturation in YPD and dilution analysis performed on SD Complete and SD-Ade and incubated at 30°C for 3 or 7 days, respectively.

2.4 DISCUSSION

In this chapter, I have investigated the genetic relationship between Rtf1, a member of the RNA polymerase II-associated Paf1 complex, and the ubiquitin ligase Rkr1. Rtf1 has strong connections to transcription, while Rkr1 has strong links to protein quality control. Although elimination of one of these factors alone does not affect cell viability, deletion of both of these factors results in synthetic lethality. Overexpression of the Rtf1-HMD, which is required for proper Rtf1-mediated histone modifications, rescues viability of *rtf1Δ rkr1Δ* strains, strengthening the genetic relationship between H2B ubiquitylation and loss of *RKR1*. To better understand the interaction between pathways of transcription and protein quality control, I used a transposon mutagenesis screen for genetic suppressors of *rtf1Δ rkr1Δ* synthetic lethality, and identified mutations in genes encoding three very different functional products (*CHL1*, *RPC17*, and *HSP104*). To understand how these genetic suppressors functioned, I investigated their ability to suppress different phenotypes and genetic interactions of *rtf1Δ* and *rkr1Δ*.

In results that will be further examined in Chapter 3, I found that deletion of *HSP104* rescued *rtf1Δ rkr1Δ* synthetic lethality by clearing cells of the prion [*PSI*$^+$], which is an aggregate of the translation termination factor Sup35 that causes increases in aberrant mRNAs and proteins. This includes an increase nonstop proteins, which are targets of Rkr1-mediated quality control (BENGTSON and JOAZEIRO 2010; DOEL *et al.* 1994; PAUSHKIN *et al.* 1996; WILSON *et al.* 2005; WILSON *et al.* 2008). Additionally, I show in this chapter that deletion of *HSP104* also rescues synthetic lethality between *rkr1Δ* and *spt10Δ*, which is further evidence that the presence of [*PSI*$^+$] negatively influences strains lacking *RKR1*. This suggests that the presence of [*PSI*$^+$] and excess nonstop proteins are detrimental in the absence of *RKR1*. However, I also found that deletion of *HSP104* slightly suppressed a sick phenotype of *rtf1Δ*

htz1Δ or *rtf1Δ arg82Δ* strains. Interestingly, mutation of *HTZ1* or genes functionally connected to *ARG82* also cause an increase in nonstop proteins, similar to *RKR1* (WILSON *et al.* 2007). These data suggest that [*PSI*$^+$] has a general effect on strains with increased nonstop protein levels. In addition, I showed that loss of *HSP104* caused a slight suppression of *rtf1Δ* 6AU sensitivity, which is a phenotype associated with defects in transcription elongations (EXINGER and LACROUTE 1992; SQUAZZO *et al.* 2002). Hsp104 is required for both protein disaggregation and prion propagation. It is unclear from these results which function of Hsp104 alleviates the *rtf1Δ* phenotypes and genetic interactions, but these data suggest the possibility that the presence of prions could also negatively affect some defects in *rtf1Δ* strains.

The Paf1 complex is required to prevent aberrantly formed transcripts that are targets of nonsense-mediated decay (PENHEITER *et al.* 2005). It has also been shown that Paf1 complex members are required for the expression or stability of nonsense and nonstop transcripts from both endogenous loci and reporter plasmids (PENHEITER *et al.* 2005; STRAWN *et al.* 2009). A screen for mutations that suppressed the [*PSI*$^+$]-mediated nonsense suppression of an *ade1-14* allele identified a mutation in *CTR9* of the Paf1 complex and suggested that mutations in *CTR9* result in transcripts that may be unstable or unable to be properly translated (STRAWN *et al.* 2009). These results are supported by my data, where I have demonstrated that the loss of Paf1 complex member Rtf1 results in decreased protein product from two nonstop reporters and the *ade1-14* allele (See also Chapter 3). Further, I have shown here that a mutation in *CHL1* opposes this Rtf1-specific defect, indicating an exciting mechanism of suppression independent of [*PSI*$^+$]. Mutations in *CHL1* result in defects in sister chromatid cohesion, but also in telomeric silencing and rDNA recombination (DAS and SINHA 2005). My results suggest that this putative DNA helicase may have additional underappreciated roles in transcription. Interestingly, Rtf1-

mediated H2B ubiquitylation is also required for Dam1 methylation at kinetochores, implicating this histone modification in mitosis (LATHAM *et al.* 2011). Future studies will expand on the connections between these processes.

As demonstrated by the above findings and others, Rtf1 is most likely required for proper levels and processing of specific transcripts. In fact, errors in Paf1c have been found to cause defects in 3' end formation, including altered poly(A) site usage and shorter poly(A) tails (MUELLER *et al.* 2004; NORDICK *et al.* 2008a; PENHEITER *et al.* 2005). Therefore, it is reasonable to further hypothesize from my described results that the loss of *RTF1* causes an increase in aberrant mRNA that is lethal in combination with other quality control problems, such as those that occur in the absence of *RKR1* and presence of [*PSI*$^+$]. To address this question, I asked if a disruption in mRNA quality control, which would also cause an increase in aberrant mRNAs, could mimic *rtf1Δ rkr1Δ* synthetic lethality. Of the mutations tested, I found that loss of *XRN1* caused synthetic sickness or lethality with loss of *RKR1*. Xrn1 is required for decapping and degrading multiple aberrant transcripts by its 5' → 3' exonuclease activity, including a class of noncoding RNAs as well as some nonsense transcripts, which possess a premature termination codon (VAN DIJK *et al.* 2011) (reviewed in (FASKEN and CORBETT 2005)). Loss of *UPF1*, which is also responsible for nonsense transcript decay, was not lethal in combination with *rkr1Δ*. These results therefore do not suggest that it is one particular class of aberrant mRNAs that may cause lethality in *rkr1Δ* [*PSI*$^+$] cells. Further genetic studies into possible mechanisms of *rkr1Δ xrn1Δ* suppression may help explain if this synthetic interaction is indeed similar to that observed for *rtf1Δ rkr1Δ* strains.

Lastly, the role of my last suppressor mutation in *RPC17* is the least clear. I found that a mutation in *RPC17* caused a slight suppression of *rtf1Δ* 6AU sensitivity. The finding that an

rpc17 mutation could rescue *rtf1Δ* 6AU sensitivity suggests a connection between RNA Pol III transcription and RNA Pol II transcription elongation defects. Presumably, decreased levels of *RPC17* transcripts from an *rpc17-DAmP* allele impair RNA Pol III function, an effect that would most likely be felt at the level of translation in the cell. It is unknown if loss of *RTF1* also has an effect on translation. However, given these findings, future experiments into this function may help elucidate these results and illuminate a novel connection between Rtf1 and translation.

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Table 2: *S. cerevisiae* strains used in Chapter 2

Strain	Genotype
KY307	*MATα his3Δ200 ura3-52 trp1Δ63 lys2Δ202*
KY958	*MATα rtf1Δ101:LEU2 his3Δ200 leu2Δ1 ura3Δ0 trp1Δ63 lys2-128δ*
KY1008	*MAT**a** leu2Δ1 his4-912δ*
KY1019	*MAT**a** leu2Δ1 his4-912δ kys2-128δ*
KY1030	*MATα his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY1663	*MATα rtf1Δ102::ARG4 rkr1Δ::KanMX leu2Δ1 ura3-52 arg4-12 [pKA69: RTF1/URA3/C/A]*
KY1898	*MATα chl1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY1899	*MAT**a** chl1Δ::KanMX his3Δ200 leu2Δ1 ura3-52*
KY2161	*MAT**a** rtf1Δ101:LEU2 leu2Δ1/0 his4-912δ kys2-128δ*
KY2162	*MAT**a** chl1Δ::KanMX rtf1Δ101:LEU2 leu2Δ1/0 his4-912δ kys2-128δ*
KY2163	*MAT**a** chl1Δ::KanMX leu2Δ1/0 his4-912δ kys2-128δ*
KY2164	*MAT**a** hsp104Δ::KanMX rtf1Δ101:LEU2 leu2Δ1/0 his4-912δ kys2-128δ*
KY2165	*MAT**a** hsp104Δ::KanMX leu2Δ1/0 his4-912δ kys2-128δ*
KY2202	*MAT**a** rkr1Δ::HIS3 his3Δ200 leu2Δ1 ura3-52*
KY2205	*MAT**a** rtf1Δ102::ARG4 rkr1Δ::KanMX leu2Δ1 ura3-52 arg4-12 trp1Δ63 [pKA69: RTF1/URA3/C/A]*
KY2206	*MAT**a** chl1Δ::KanMX rtf1Δ101::LEU2 rkr1Δ::HIS3 his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY2207	*MAT**a** chl1Δ::KanMX rtf1Δ101::LEU2 his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY2208	*MATα chl1Δ::KanMX rkr1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 trp1Δ63 arg4-12*
KY2209	*MATα rtf1Δ::KanMX rkr1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 trp1Δ63* [*psi*$^-$]
KY2210	*MAT**a** rtf1Δ::KanMX rkr1Δ::KanMX his3Δ200 leu2Δ1 trp1Δ63* [*psi*$^-$]
KY2212	*MAT**a** ade1-14 leu2Δ1 ura3-52 trp1Δ63*
KY2213	*MAT**a** rtf1Δ101::LEU2 ade1-14 leu2Δ1 ura3-52 trp1Δ63*
KY2214	*MATα chl1Δ::KanMX ade1-14 leu2Δ1 ura3-52 trp1Δ63*
KY2215	*MATα chl1Δ::KanMX rtf1Δ101::LEU2 ade1-14 leu2Δ1 ura3-52 trp1Δ63*
KY2236	*MATα rkr1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KA137	*MATα rpc17-DAmP::KanMX his3Δ1/200 leu2Δ0/1 lys2-128δ ura3Δ0/-52 trp1Δ63*
OKA248	*MAT**a** rtf1Δ101::LEU2 rpc17-DAmP::KanMX leu2Δ0/1 his4-912δ kys2-128δ*
OKA249	*MATα rpc17-DAmP::KanMX leu2Δ0/1 his4-912δ kys2-128δ*
SHY13	*MAT**a** rkr1Δ::HIS3 his3Δ200 leu2Δ1 ura3-52 lys2-128δ*
KMKY174	*MATα rkr1Δ::HIS3 rpc17-DAmP::KanMX his3Δ1/200 leu2Δ0/1 ura3Δ0/-52*
KMKY178	*MATα rtf1Δ101::LEU2 rpc17-DAmP::KanMX his3Δ1/200 leu2Δ0/1 ura3Δ0/-52 lys2-128δ*
KMKY181	*MATα rtf1Δ101::LEU2 his3Δ1/200 leu2Δ0/1 ura3Δ0/-52 lys2-128δ trp1Δ63 met15Δ0*

3.0 THE PAF1 COMPLEX SUBUNIT RTF1 BUFFERS CELLS AGAINST THE TOXIC EFFECTS OF [*PSI*$^+$] AND DEFECTS IN RKR1-DEPENDENT QUALITY CONTROL IN *S. CEREVISIAE*

3.1 INTRODUCTION

During transcription elongation, various proteins modify chromatin in coordination with RNA polymerase (RNA Pol II) to ensure accurate and efficient transcription of nucleosomal templates (LI *et al.* 2007a). Changes in chromatin include nucleosome remodeling, the exchange of histone variants for canonical histones, and histone modifications such as the methylation, ubiquitylation, and acetylation of lysine (K) residues. The conserved Paf1 complex (Paf1c), which consists of Paf1, Ctr9, Leo1, Cdc73, and Rtf1, associates with RNA Pol II on all actively transcribed genes (MAYER *et al.* 2010) and couples the modification of histones to transcription elongation (reviewed in (CRISUCCI and ARNDT 2011b; JAEHNING 2010)). Paf1c is required for multiple histone modifications associated with active genes including the monoubiquitylation of H2B K123, a modification for which the Rtf1 subunit of Paf1c plays a prominent role (NG *et al.* 2003a; TOMSON *et al.* 2011; WARNER *et al.* 2007; WOOD *et al.* 2003b). Rad6 is the ubiquitin conjugating enzyme (E2) for H2B K123 ubiquitylation, while Bre1 is the ubiquitin-protein ligase (E3) (HWANG *et al.* 2003). This modification is a prerequisite for downstream histone H3

methylation (DOVER *et al.* 2002; NG *et al.* 2003a; SUN and ALLIS 2002; WOOD *et al.* 2003b). In yeast, loss of H2B K123 ubiquitylation broadly impacts gene expression and chromatin structure (BATTA *et al.* 2011; MUTIU *et al.* 2007). In humans, errors in Paf1c-dependent histone modifications can lead to aberrant gene expression and tumorigenesis (reviewed in (CRISUCCI and ARNDT 2011b)).

Paf1c has several functions in addition to promoting specific histone modifications, including directing the proper 3'-end formation of transcripts (MUELLER *et al.* 2004; NAGAIKE *et al.* 2011; NORDICK *et al.* 2008a; PENHEITER *et al.* 2005; SHELDON *et al.* 2005). Depletion of human Paf1c (hPaf1c) subunits impairs mRNA cleavage, polyadenylation, and export to the cytoplasm (NAGAIKE *et al.* 2011). Additionally, loss of hCdc73 results in aberrantly processed and polyadenylated histone mRNAs (FARBER *et al.* 2010). In yeast, deletion of Paf1c subunits leads to decreased poly(A) tail length and alternative poly(A) site usage (MUELLER *et al.* 2004; STRAWN *et al.* 2009). These observations indicate that Paf1c is essential for both the proper expression and processing of a subset of RNAs and that loss of Paf1c can result in aberrant transcripts, which are inefficiently exported or translated. In support of this idea, erroneous transcripts resulting from loss of Paf1c are substrates for mRNA quality control pathways, including nonsense mRNA decay (PENHEITER *et al.* 2005) (reviewed in (CRISUCCI and ARNDT 2011b; JAEHNING 2010)).

RKR1, which is required for viability in *S. cerevisiae* strains lacking the Paf1c subunit Rtf1, encodes a conserved RING finger-containing ubiquitin-protein ligase (BRAUN *et al.* 2007). Deletion of *RKR1* also causes severe growth defects in strains lacking *PAF1* or *CTR9* (BRAUN *et al.* 2007). Given that *rkr1Δ* causes severe synthetic growth defects in strains with an *htb1-K123R* mutation, Rkr1 most likely functions in a pathway parallel to the histone modification functions

of Paf1c to promote an important cellular process (BRAUN *et al.* 2007). Interestingly, Rkr1 is required for the proper ubiquitylation and degradation of nonstop proteins in yeast and physically associates with ribosomes (BENGTSON and JOAZEIRO 2010; BRAUN *et al.* 2007; FLEISCHER *et al.* 2006; WILSON *et al.* 2007). Nonstop proteins can result from mRNAs lacking stop codons (nonstop mRNAs), and both the mRNAs that encode these nonstop proteins and the resulting nonstop proteins themselves are targeted for degradation (BENGTSON and JOAZEIRO 2010; VAN HOOF *et al.* 2002; WILSON *et al.* 2007), thus implicating Rkr1 in a protein quality control pathway (BENGTSON and JOAZEIRO 2010). Importantly, mutations in *RKR1* homologs in higher eukaryotes are associated with neurodegeneration and colon cancer (CHU *et al.* 2009; IVANOV *et al.* 2007).

To investigate the relationship between the transcription factor Rtf1 and the protein quality control factor Rkr1, I performed a transposon-based mutagenesis screen to identify suppressors of *rtf1Δ rkr1Δ* synthetic lethality in *S. cerevisiae*. I found that mutations in the gene encoding the Hsp104 chaperone rescue lethality of an *rtf1Δ rkr1Δ* strain. Enhanced or depleted levels of Hsp104 alter [*PSI*$^{+}$] prion propagation (CHERNOFF *et al.* 1995), suggesting a role for this prion in *rtf1Δ rkr1Δ* synthetic lethality. Consistent with this idea, I found that conditions for curing [*PSI*$^{+}$] restore viability of *rtf1Δ rkr1Δ* strains and that transfer of [*PSI*$^{+}$] to *rtf1Δ rkr1Δ* [*psi*$^{-}$] cells causes lethality. Additionally, I found that the presence of [*PSI*$^{+}$], presumably through increased nonstop proteins, negatively influences the fitness of *rkr1Δ* strains even in the presence of Rtf1. Unexpectedly, the absence of *RTF1* in a *rkr1Δ* background causes a decrease in the levels of nonstop reporter proteins. My results suggest that Rtf1 and its H2B ubiquitylation function protect cells against the combined deleterious effects of [*PSI*$^{+}$] and defects in Rkr1-mediated protein quality control.

3.2 MATERIALS AND METHODS

3.2.1 Yeast strains and standard growth conditions

KY *Saccharomyces cerevisiae* strains are isogenic with FY2, a *GAL2*$^{+}$ derivative of S288C (WINSTON *et al.* 1995). Yeast deletion mutants, crosses, and transformants were created using standard protocols (AUSUBEL 1988; ROSE 1991). Yeast were grown on rich (YPD), synthetic complete (SC), synthetic minimal (SD), 5-fluoro-orotic acid (5-FOA) or sporulation media as specified and prepared as previously described (ROSE 1991). Strains were typically cured of prions by streaking for single colonies onto YPD supplemented with 5 mM guanidine hydrochloride. For creating [prion-] strains from diploids, tetrad dissections were performed on YPD containing 2.5 mM guanidine hydrochloride.

3.2.2 Plasmids

The *his3* nonstop plasmid, pAV240 (*LEU2*-marked), and protein A nonstop plasmid, pAV184 (*URA3*-marked), were gifts from Dr. Ambro van Hoof (WILSON *et al.* 2007). The *sup35NM*-GFP (*URA3*- or *LEU2*-marked) and *RNQ1*-GFP plasmids were gifts from Dr. Susan Liebman (ZHOU *et al.* 2001). The *URA3*-marked plasmid carrying *RTF1*, pKA69, was used to maintain *rtf1Δ rkr1Δ* viability (STOLINSKI *et al.* 1997). *HSP104* was driven by a GPD promoter on a 2μ pRS424 (*TRP1*) plasmid (MUMBERG *et al.* 1995; RUBEL *et al.* 2008). This plasmid was used to derive

plasmids for overexpression of *URE2*, *LSM4*, *LSM2*, and *RNQ1*. The open reading frames and 3' UTR sequences of *URE2*, *LSM4*, *LSM2*, and *RNQ1* were amplified by PCR from a plasmid source and inserted in place of *HSP104* using the *Sac*I and *BamH*I sites (NAGALAKSHMI *et al.* 2008). *LSM4* and *URE2* overexpression clones were made by Mark Sullivan. The *ade1-14* and *sup35-Y351C* alleles (BRADLEY *et al.* 2003) were amplified from strains provided by Dr. Susan Liebman and cloned using *Xma*I and *Sac*I sites into pRS306 for two-step gene replacement of the *ADE1* or *SUP35* gene, respectively (SCHERER and DAVIS 1979).

3.2.3 Mutagenesis and confirmation of genetic suppressors

Transposon (Tn) mutagenesis to identify suppressors of *rtf1Δ rkr1Δ* synthetic lethality was performed by transforming a *LEU2*-marked set of integrating plasmids (described in (KUMAR *et al.* 2000)) into an *rtf1Δ rkr1Δ* strain (KY1663) carrying an *RTF1/URA3/CEN/ARS* plasmid (pKA69) and selecting on SC-leucine (L) medium, as described in detail in section 2.2.4 of this thesis.

3.2.4 High-copy-number suppressor screen

To obtain high-copy-number suppressors of *rtf1Δ rkr1Δ* synthetic lethality, a 2-micron *LEU2*-marked plasmid library of genomic fragments (RUBEL *et al.* 2008; YOSHIHISA and ANRAKU 1989) was transformed into an *rtf1Δ rkr1Δ* strain (KY2205) carrying pKA69. Approximately 13,500 Leu^+ colonies were screened for the ability to grow on 5-FOA medium, indicating loss of pKA69. One hundred and thirty-one candidates were verified by testing on 5-FOA medium, and plasmids were isolated by standard extraction methods (HOFFMAN and WINSTON 1987). Plasmids

were then re-transformed into KY2205, and 48 exhibited the suppression phenotype. Of these, 21 contained either *RTF1* or *RKR1*. Of the remaining candidates, six unique plasmids remained. One plasmid, which was isolated seven times, contained the gene *LSM4*. Another plasmid, which was isolated three times, contained multiple open reading frames, including *URE2*. A third plasmid, obtained twice, also contained multiple open reading frames, including *HSP104*. This screen was originally performed by Mary Braun.

3.2.5 Yeast dilution growth assays

Unless stated otherwise, yeast strains were grown to saturation at 30^{o}C in rich or selective media, washed with sterile water, and diluted into 1x10^{8} cells/ml stocks from which 10-fold dilutions were made. Two microliters of cell suspension were plated on appropriate control and selective media and plates were incubated at 30^{o}C for the specified number of days. Media for testing *rtf1Δ* and *rkr1Δ* phenotypes contained 0.8 µg/ml cycloheximide, 50 µM cadmium chloride, 10% ethanol, or 15 mM caffeine in YPD or SC as indicated.

3.2.6 Cytoduction

An *rtf1Δ rkr1Δ ade1-14* [*psi*$^{-}$] strain (KY2286), created by dissection onto YPD containing GuHCl, was transformed with *RTF1/URA3* (pKA69) and made ρ^{0} by growth in liquid culture with ethidium bromide. Using previously described methods (WICKNER *et al.* 2006), this recipient strain was used for cytoduction with two *kar1* donor strains, L2261 ([*PIN*$^{+}$] [*psi*$^{-}$]) and L2265 ([*PSI*$^{+}$] [*pin*$^{-}$]) (MATHUR *et al.* 2009). Transfer of cytoplasm to the recipient strain was confirmed by growth on YP medium containing 3% glycerol (YPG) and transfer of the donor

prion was confirmed by live cell confocal microscopy of plasmid-encoded GFP-tagged prion domains. The *kar1* donor strains and GFP plasmids were gifts from Dr. Susan Liebman.

3.2.7 Live cell confocal microscopy

Strains were transformed with *sup35NM*-GFP plasmid (ZHOU *et al.* 2001) to test for the presence of [*PSI*$^+$] or a *RNQ1*-GFP plasmid to test for the presence of [*PIN*$^+$] and patched onto selective media containing 100 μM $CuSO_4$. Plates were protected from light and incubated at 30^{o}C for several days. Live cell imaging was performed on wet mounts using a Leica TCS SP5 Confocal Microscope (Leica Microsystems, Buffalo Grove, IL).

3.2.8 Immunofluorescence

A *rkr1Δ* strain (KY2289) was transformed with either an *HA-RKR1* (pMB11) or untagged *RKR1* plasmid (pPC65) (BRAUN *et al.* 2007). These strains were grown to mid-log phase and prepared as previously described (AMBERG *et al.* 2006). Briefly, cells were fixed with formaldehyde, treated with Zymolyase 20T, and adhered to a polylysine slide before overnight incubation with 1:500 anti-HA (Roche) and 1 hour incubation with 1:250 Alexa 647 (Molecular Probes). Slides were mounted with ProLong GOLD Antifade DAPI reagent (Invitrogen) and imaged using a Leica TCS SP5 Confocal Microscope (Leica Microsystems, Buffalo Grove, IL).

3.2.9 Western analysis of nonstop protein levels

Strains transformed with pAV184 (WILSON *et al.* 2007) were grown at 30°C in SC-uracil (U) liquid culture containing 2% galactose to an OD_{600} of 0.7 to 0.9. Cells were normalized to 10.8 OD_{600} units and extracts were made using glass bead lysis in 20% trichloroacetic acid as previously described (COX *et al.* 1997; ZHENG *et al.* 2010). An equal amount of each extract (5 µl) was run on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose membrane for western analysis using standard methods (HARLOW and LANE 1988). The membrane was probed with Peroxidase-Anti-Peroxidase (1:2000 dilution; Sigma) to assay levels of Protein A and anti-G6PDH antibody (1:50,000 dilution; Sigma) as a loading control. Immunoreactivity was measured using chemiluminescence (Perkin-Elmer) and a 440 CF digital imaging station (Kodak).

3.3 RESULTS

3.3.1 Genetic suppressors of *rtf1*Δ *rkr1*Δ synthetic lethality

To investigate the basis for lethality of strains lacking Rtf1 and the ubiquitin-protein ligase Rkr1, I performed a transposon-based (Tn) mutagenesis screen for suppressors of the *rtf1Δ rkr1Δ* synthetic lethal interaction as described in Chapter 2 of this thesis. One candidate that met these criteria is described in further detail in this chapter, while two additional candidates are also described in Chapter 2.

Following the plasmid rescue of the transposon insertion (BURNS *et al.* 1994), DNA sequencing revealed a transposon insertion in the 3' coding region of *HSP104*. Hsp104 encodes a heat shock protein that can disrupt aggregated proteins (PARSELL *et al.* 1994) and is involved in the maintenance and propagation of yeast prions (reviewed in (GRIMMINGER-MARQUARDT and LASHUEL 2010)). As described in Chapter 2, to confirm that the Tn mutation rescued *rtf1*Δ *rkr1*Δ synthetic lethality by disrupting Hsp104 function, I generated an *hsp104Δ* strain and crossed it to a *rkr1Δ* strain. Double mutants from these crosses were then mated with an *rtf1Δ* strain. The diploids, which are heterozygous for three genes (*rtf1*Δ, *rkr1*Δ, and *hsp104*Δ), were subjected to tetrad analysis. Surprisingly, the *rtf1*Δ *rkr1*Δ double mutant segregants were alive and healthy, indicating dominant suppression by *hsp104*Δ (Figure 15A). In addition, the *hsp104Δ rtf1Δ rkr1Δ* triple mutants were viable, independently confirming the identification of an *hsp104* mutation in my suppressor screen (Figure 15A).

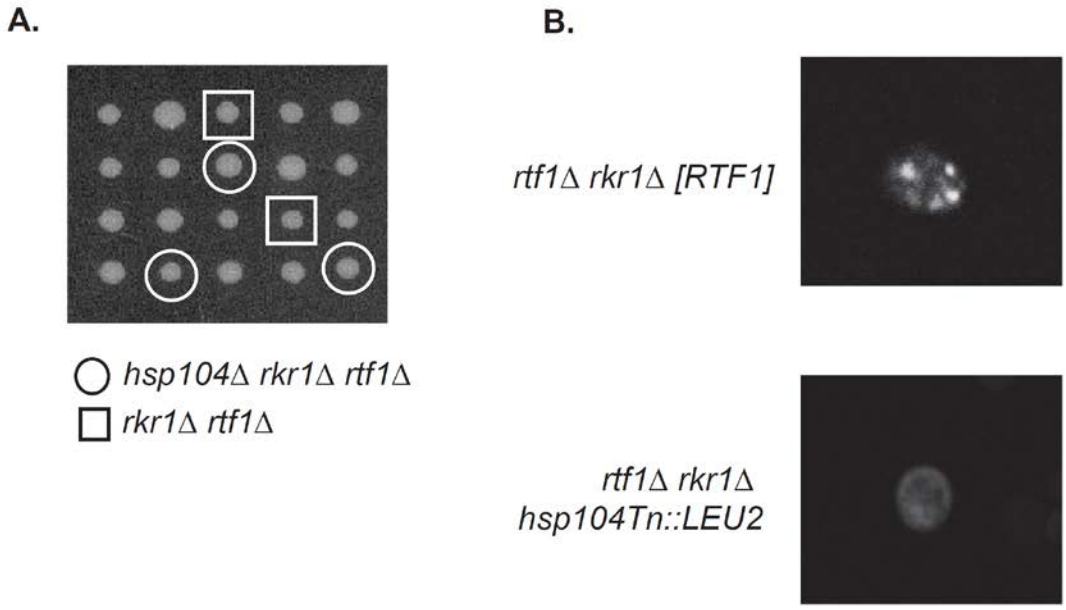

Figure 15: Mutation of *HSP104* suppresses *rtf1Δ rkr1Δ* synthetic lethality and cures [*PSI*$^+$]

This figure is a combination of results shown in Chapter 2. (A) Tetrad dissections of crosses between an *rtf1Δ* strain (KY958) and a *rkr1Δ hsp104Δ* strain. Dissections were done on YPD and incubated at 30^{o}C for 3 days**.** Double mutant *rtf1Δ rkr1Δ* segregants are highlighted by boxes while triple mutants are highlighted by circles. Note suppression of *rtf1Δ rkr1Δ* synthetic lethality occurs with or without *hsp104Δ* co-segregation, indicating that *hsp104Δ* acts as a dominant suppressor in the diploid. (B) The strain originally used for transposon-mediated mutagenesis (KY1663) and the *hsp104Tn::LEU2* mutant recovered from the transposon-based suppressor screen were transformed with a *LEU2*-marked or a *URA3*-marked *pCUP1-SUP35NM-GFP* plasmid. Strains were patched onto SC-L or SC-U plates containing 100 µM $CuSO_4$ and incubated in the dark at 30^{o}C before live cell imaging was performed by confocal microscopy. Observations were made of at least three transformants per strain and 100 cells per transformant. Representative images are shown. No variability was seen among cells with respect to the GFP pattern.

3.3.2 The transposon insertion in *HSP104* cures cells of [*PSI*$^+$]

As described in Chapter 2, deletions in the C-terminal domain of Hsp104 have been shown to weaken its ATPase activity and ability to propagate prions, particularly [*PSI*$^+$], an aggregate of the translation termination factor Sup35 (CHERNOFF *et al.* 1995; MACKAY *et al.* 2008). Because the transposon insertion disrupted the C-terminal domain of Hsp104, I investigated if the *rtf1Δ rkr1Δ* strain used in the suppressor screen was [*PSI*$^+$] and whether the *hsp104Tn::LEU2* suppressor mutation cleared [*PSI*$^+$] from this strain. A plasmid expressing the GFP-tagged prion domain of Sup35 was transformed into the original *rtf1Δ rkr1Δ [RTF1, URA3, CEN/ARS]* strain used in my transposon mutagenesis screen and transformants were visualized by live-cell imaging using a confocal microscope (ZHOU *et al.* 2001). Previous studies have shown that this GFP-tagged Sup35 protein appears as small fluorescent puncta in [*PSI*$^+$] cells and as diffuse fluorescence in [*psi*$^-$] cells (ZHOU *et al.* 2001). Using this method, I found that the original strain used in our screen was indeed [*PSI*$^+$] and that a transposon insertion within *HSP104* resulted in [*psi*$^-$] conditions in all cells examined (Figure 15B). Given the importance of Hsp104 in prion propagation, I next investigated the role of prions in the genetic interaction between *RTF1* and *RKR1*.

3.3.3 *RKR1* genetic interactions are rescued by curing strains of [*PSI*$^+$]

As noted above, *HSP104* is required for the propagation of yeast prions (CHERNOFF *et al.* 1995; SHORTER and LINDQUIST 2006). Therefore, the loss of prions in an *hsp104Δ/HSP104* heterozygous diploid strain likely rescued lethality between *rtf1Δ* and *rkr1Δ,* independently of whether the *hsp104*Δ mutation actually segregated with the *rtf1*Δ and *rkr1*Δ mutations (Figure

15A). Growth on media containing guanidine hydrochloride has been shown to cure yeast of prions by inactivating the ATPase domain of Hsp104 (FERREIRA *et al.* 2001; JUNG and MASISON 2001). To determine if clearing prions through this method could also rescue *rtf1Δ rkr1Δ* lethality, an *rtf1Δ/RTF1 rkr1Δ/RKR1* heterozygous diploid was sporulated and tetrads were dissected onto YPD or YPD containing 2.5 mM guanidine hydrochloride (GuHCl). Strikingly, tetrad analysis revealed that *rtf1Δ rkr1Δ* double mutants grew as well as wild-type strains on YPD containing GuHCl but were inviable on YPD alone (Figure 16A). Therefore, inactivation of *HSP104* by mutation or treatment with GuHCl rescues *rtf1Δ rkr1Δ* synthetic lethality.

HSP104 is required for the propagation of several yeast prions, including [*PSI*$^+$], [*URE3*], and [*PIN*$^+$] (reviewed in (HASLBERGER *et al.* 2010)). Similar to a loss of *HSP104* function, overexpression of *HSP104* can also alleviate yeast of [*PSI*$^+$], but it has not been shown to affect the propagation of other yeast prions (CHERNOFF *et al.* 1995; SHORTER and LINDQUIST 2006). Therefore, to investigate if *rtf1Δ rkr1Δ* lethality might be rescued by loss of [*PSI*$^+$] or of yeast prions in general, I overexpressed *HSP104* to test for rescue of synthetic lethality. An *rtf1Δ rkr1Δ* strain, which carried an *RTF1 URA3*-marked plasmid to allow growth, was transformed with a *TRP1*-marked *HSP104* overexpression plasmid. Transformants were grown under selective conditions and replica plated to medium containing 5-FOA to select for loss of the *RTF1* plasmid. Interestingly, the *HSP104* high-copy plasmid allowed for growth of the *rtf1Δ rkr1Δ* double mutants (Figure 16B). Taken together, my results indicate that deletion, inactivation, or overexpression of *HSP104* suppresses *rtf1Δ rkr1Δ* synthetic lethality by clearing [*PSI*$^+$].

A defect in H2B K123 ubiquitylation can phenocopy an *rtf1Δ* mutation with respect to *rkr1Δ* synthetic growth defects (BRAUN *et al.* 2007). I therefore investigated if the inactivation of

Hsp104 could also rescue the genetic interaction between *rkr1Δ* and *htb1-K123R*, a derivative of H2B that lacks the ubiquitylation site for Rad6-Bre1. To answer this question, I performed a plasmid shuffle experiment with *RKR1* and *rkr1Δ* strains, which carried a *URA3*-marked *HTA1-HTB1* plasmid and a *HIS3*-marked *HTA1-htb1K123R* plasmid, and were deleted for the chromosomal H2A and H2B genes. Serial dilution analysis on 5-FOA medium selected for cells that had lost the *URA3*-marked wild-type *HTA1-HTB1* plasmid and retained the *HIS3*-marked *HTA1-htb1K123R* plasmid. As previously shown, the *rkr1Δ* mutation causes a strong synthetic growth defect in combination with the H2B K123R substitution by this assay (Figure 16C) (BRAUN *et al.* 2007). However, if these strains were first cured of prions by passaging on medium containing 5 mM GuHCl prior to plating on 5-FOA medium, *rkr1Δ htb1K123R* strains were viable (Figure 16C).

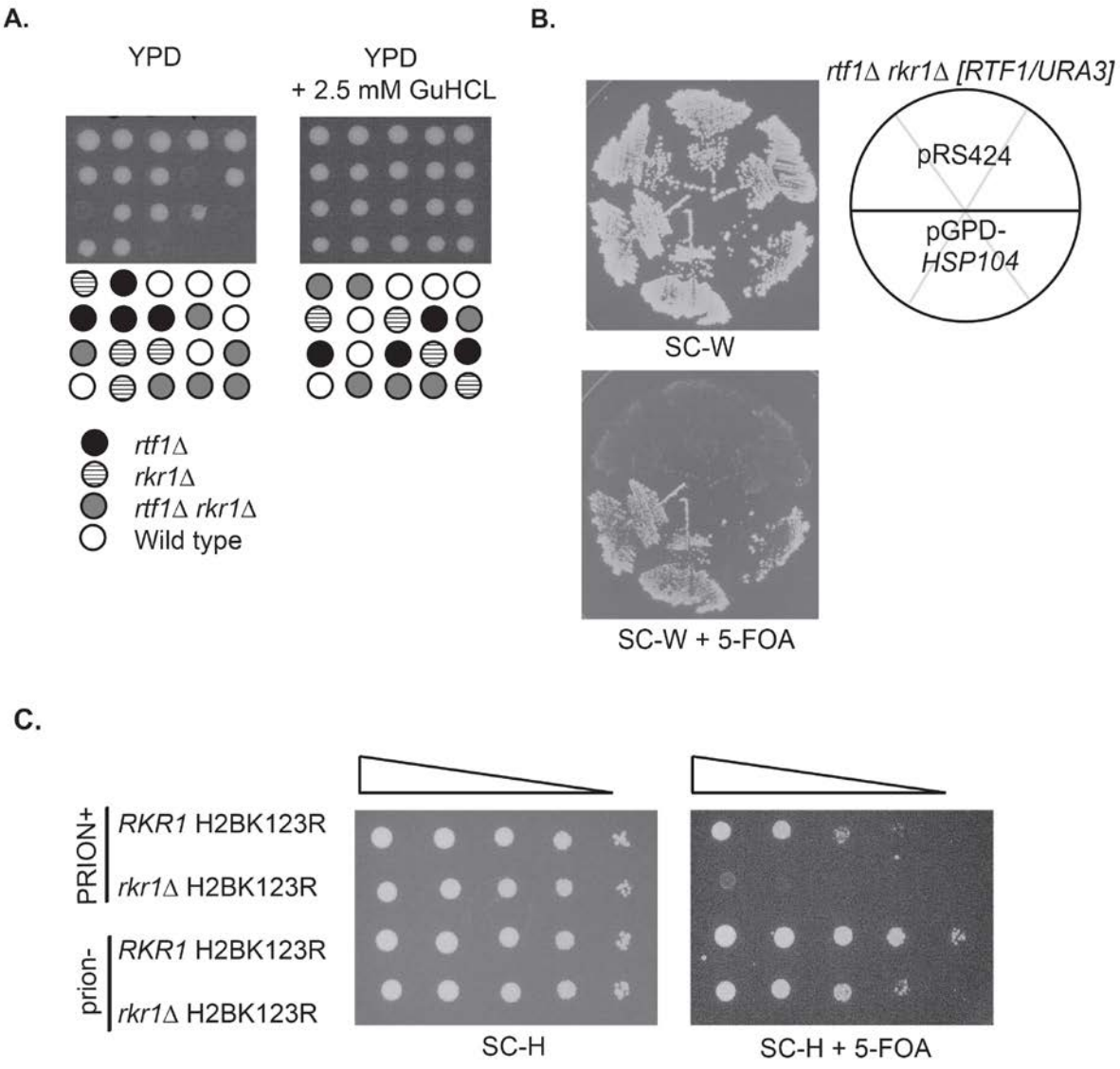

Figure 16: Inactivation or overexpression of *HSP104* rescues *rkr1Δ* synthetic genetic interactions

(A) Heterozygous *rtf1Δ/RTF1 rkr1Δ/RKR1* diploids (KY2202 mated by KY453) were dissected onto YPD or YPD containing 2.5 mM guanidine hydrochloride (GuHCl) and incubated at 30°C for 3 days. (B) An *rtf1Δ rkr1Δ [RTF1/URA3]* strain (KY1663) was transformed with either a 2μ *TRP1*-marked *pGPD-HSP104* plasmid or a *TRP1*-marked empty vector (pRS424), and transformants were purified on SC-W and replica plated to SC-W + 5-FOA.

Plates were incubated at 30°C for 3 days. (C) Wild type (KY2203) or *rkr1Δ* (KY2204) strains, lacking both endogenous histone H2A and H2B gene copies, were transformed with a *URA3*-marked wild type copy of *HTA1-HTB1* and a *HIS3*-marked *HTA1-htb1 K123R* plasmid. Strains were cured by streaking onto YPD + 5 mM GuHCl. Dilution growth assays were performed on SC-H or SC-H + 5-FOA, and cells were incubated at 30°C for 2 days. PRION+ indicates uncured cells; prion- indicates cells passaged on medium containing GuHCl.

3.3.4 Overexpression of prion-coding genes *URE2* and *LSM4* rescues *rtf1Δ rkr1Δ* lethality

Over twenty potential or verified prions have been identified in budding yeast, with [*PSI*$^+$] being one of the best characterized (ALBERTI *et al.* 2009). Interactions between different prions can be both positive and negative. The *de novo* formation and propagation of some prions require the presence of other prions (DERKATCH *et al.* 2001), while the maintenance of some prions may be negatively impacted by the presence of other prions, possibly by affecting chaperone activity (reviewed in (CROW and LI 2011)).

In a second, unbiased suppression screen (see Material and Methods), Mary Braun identified high-copy-number plasmids that rescued *rtf1Δ rkr1Δ* synthetic lethality. Confirming earlier results, one plasmid suppressor contained *HSP104*. Interestingly, several other suppressor plasmids contained *URE2*, which encodes the prion [*URE3*] (reviewed in (MASISON *et al.* 2000)), or *LSM4*, which codes for a protein with a prion-forming domain (ALBERTI *et al.* 2009). The overexpression of [*URE3*] has been shown to antagonize the propagation of [*PSI*$^+$] (SCHWIMMER and MASISON 2002). Therefore, to further test the hypothesis that the clearance of [*PSI*$^+$] suppresses *rtf1Δ rkr1Δ* lethality, I transformed an *rtf1Δ rkr1Δ* double mutant strain

carrying an *RTF1 URA3*-marked plasmid with overexpression plasmids for the prions [*URE3*] or [*LSM4*] and assessed the growth of these strains on 5-FOA medium (ALBERTI *et al.* 2009; DERKATCH *et al.* 2001). *HSP104* overexpression served as a positive control. As expected, the *rtf1Δ rkr1Δ* strain was unable to grow without a plasmid-source of *RTF1*. However, the overexpression of prion-encoding genes *URE2* and *LSM4* suppressed *rtf1Δ rkr1Δ* synthetic lethality to the same degree as overexpression of *HSP104* (Figure 17A). I also investigated the effects of overexpressing the gene *RNQ1*, which encodes the prion [*PIN*$^+$] (DERKATCH *et al.* 2001), on *rtf1Δ rkr1Δ* synthetic lethality. Although some transformants revealed suppression of *rtf1Δ rkr1Δ* synthetic lethality by *RNQ1* overexpression, others did not, possibly because [*PSI*$^+$] and [*PIN*$^+$] variants can differentially affect each other's propagation (Figure 18) (BRADLEY and LIEBMAN 2003).

Collectively, my results indicate that [*PSI*$^+$] is negatively impacted by overexpression of [*URE3*] and [*LSM4*] prions, and that [*PSI*$^+$] causes *rtf1Δ rkr1Δ* mutants to be inviable. To investigate if the aggregation propensity of [*PSI*$^+$] is negatively affected under each of these conditions, I used live-cell confocal microscopy to image the presence of [*PSI*$^+$] in strains overexpressing *HSP104*, *URE2*, or *LSM4*. As expected, *rtf1Δ rkr1Δ [RTF1, URA3, CEN/ARS]* cells expressing the Sup35NM-GFP 2-micron plasmid contained fluorescent puncta confirming the presence of [*PSI*$^+$] (Figure 17B). In strains that overexpressed Sup35NM-GFP and also *HSP104, URE2, or LSM4*, I observed a large number of cells that exhibited diffuse fluorescence and were apparently cured of [*PSI*$^+$] (Figure 17B and 17C).

The recovery of *URE2* and *LSM4* from an independent genetic screen as suppressors of *rtf1Δ rkr1Δ* lethality further indicated the importance of [*PSI*$^+$] to this genetic interaction. However, due to the known roles of Lsm4 in regulating RNA processing and degradation as part

of the Lsm complex (reviewed in (BEGGS 2005), I asked whether the suppression of *rtf1Δ rkr1Δ* lethality by *LSM4* overexpression could be explained by a disruption of Lsm complex function. To this end, I constructed a plasmid to overexpress another member of the Lsm complex, Lsm2, which is not known to have a prion-forming domain. Unlike the case for *LSM4*, the *LSM2* overexpression plasmid did not rescue *rtf1Δ rkr1Δ* synthetic lethality (Figure 18). Collectively, my data indicate a positive correlation between overexpression of certain prions, clearance of [*PSI*$^{+}$], and suppression of *rtf1Δ rkr1Δ* inviability.

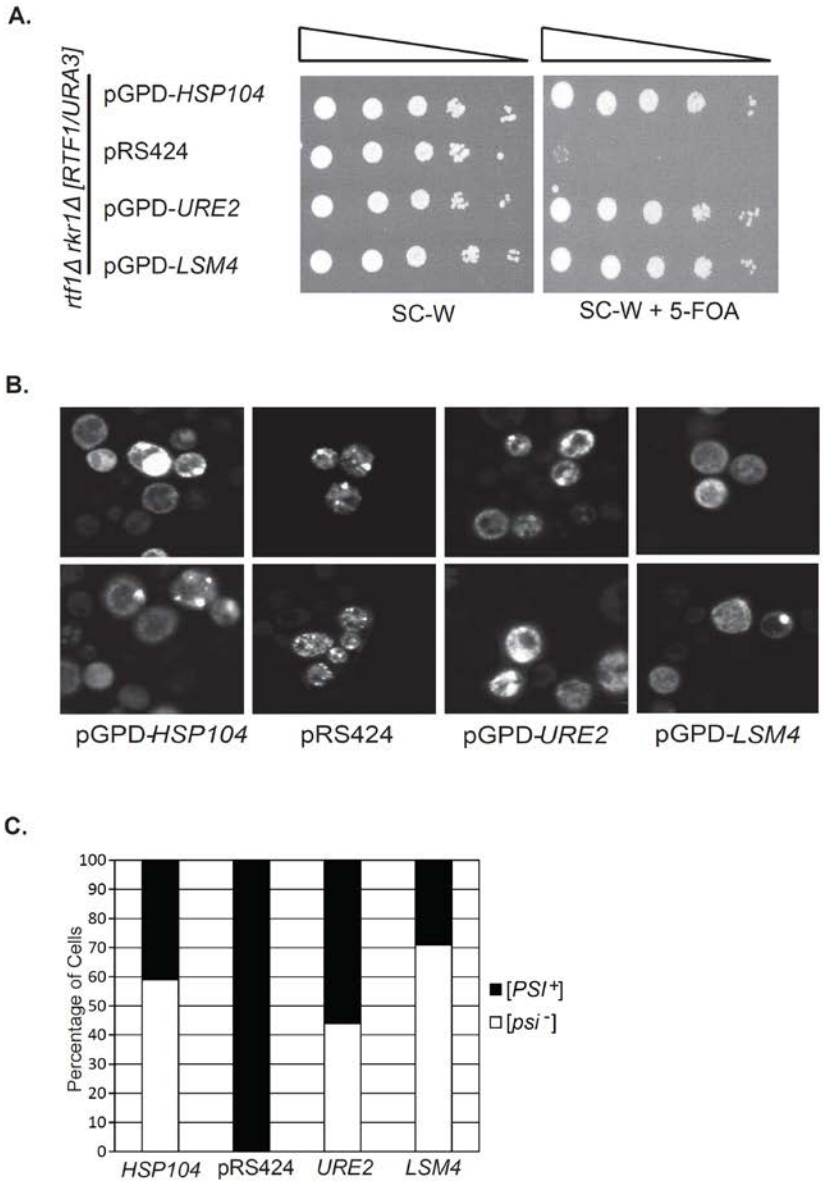

Figure 17: Overexpression of *HSP104, URE2* or *LSM4* rescues *rtf1Δ rkr1Δ* lethality and clears [*PSI*$^+$]

(A) An *rtf1Δ rkr1Δ* strain (KY2205) carrying a *URA3*-marked *RTF1* plasmid was transformed with 2µ *TRP1*-marked p*GPD-HSP104*, p*GPD-URE2*, p*GPD-LSM4*, or empty vector. Dilution assays were performed on SC-W or SC-W + 5-FOA and plates were incubated at 30^{o}C for 2 days. (B) Strains from (A) were transformed with *LEU2*-marked p*CUP1-SUP35NM-GFP*. Transformants were patched onto SC-L-W containing 100 µM $CuSO_4$ and incubated in the dark at 30^{o}C before live cell imaging was performed using confocal microscopy. Two

representative images are shown for each strain. (C) This panel displays the percentage of [*PSI*$^{+}$] and [*psi*$^{-}$] cells from among 50-100 cells from three separate transformants.

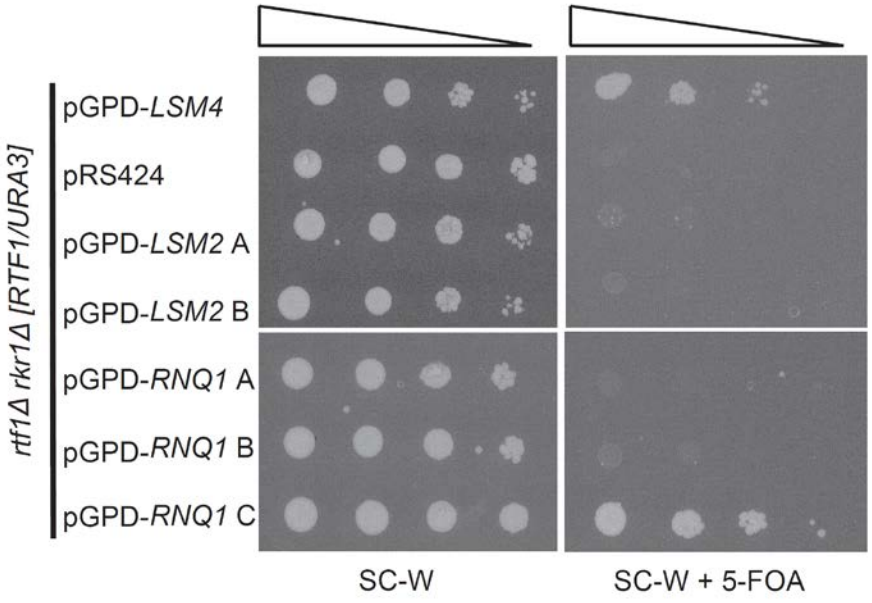

Figure 18: Effect of *LSM2* or *RNQ1* overexpression on *rtf1Δ rkr1Δ* synthetic lethality

An *rtf1Δ rkr1Δ* strain (KY2205) carrying a *URA3*-marked *RTF1* plasmid was transformed with 2µ *TRP1*-marked p*GPD-LSM4*, p*GPD-LSM2*, p*GPD-RNQ1*, or empty vector. Ten-fold serial dilution assays were performed on SC-W or SC-W + 5-FOA media and plates were incubated at 30^{o}C for 2 days. Overexpression of *RNQ1* resulted in 9 out of 23 transformants that showed rescue of *rtf1Δ rkr1Δ* lethality (representative transformants depicted as A, B, or C). No variation in the lack of rescue of *rtf1Δ rkr1Δ* lethality by overexpression of *LSM2* was observed in 25 transformants (representative transformants depicted as A or B).

3.3.5 [*PSI*$^+$] causes *rtf1Δ rkr1Δ* synthetic lethality

To confirm that the combined loss of *RTF1* and *RKR1* causes synthetic lethality only in [*PSI*$^+$] conditions, I performed cytoduction experiments using a viable, cured *rtf1Δ rkr1Δ* strain transformed with a *URA3*-marked *RTF1* plasmid as a recipient strain and specific prion-containing donor strains. The recipient also carried the *ade1-14* nonsense allele to monitor the transfer of [*PSI*$^+$], which causes translational read-through of *ade1-14* and production of functional Ade1 protein (CHERNOFF *et al.* 1995). Cytoductions were performed with two donor strains (MATHUR *et al.* 2009), one carrying [*PSI*$^+$] and the other carrying [*PIN*$^+$]. Only cytoduction of [*PSI*$^+$] resulted in the inability to lose the *RTF1* plasmid, as indicated by growth on SD-Ade and death on 5-FOA media (Figure 19A). I confirmed transfer of [*PSI*$^+$] or [*PIN*$^+$] by live-cell confocal microscopy with GFP tagged-prion domains (Figure 19B). These results confirm that the prion [*PSI*$^+$] causes *rtf1Δ rkr1Δ* lethality.

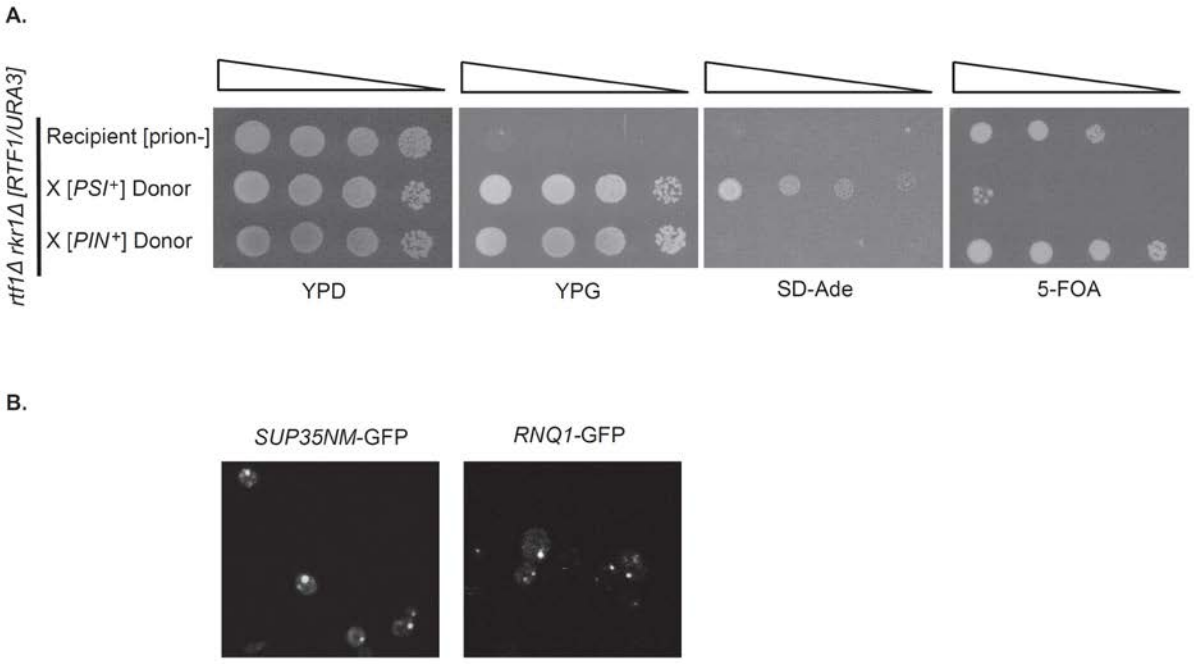

Figure 19: Cytoduction of [*PSI*$^+$] into cured *rtf1Δ rkr1Δ* strains causes synthetic lethality

A) An *rtf1Δ rkr1Δ ade1-14* strain (KY2286) cured of prions by dissection on medium containing GuHCl was transformed with a *URA3*-marked *RTF1* plasmid and made ρ^0 by growth in ethidium bromide-containing medium. This strain served as a recipient for cytoduction with donor *kar1* strains containing only [*PSI*$^+$] (L2265) or [*PIN*$^+$] (L2261). A dilution growth assay is shown with the recipient, cytoductants, and donors on YPD, YPGlycerol (YPG) to show successful cytoduction of recipients, SD-Ade to show transfer of [*PSI*$^+$], and 5-FOA to score for loss of the *RTF1/URA3* plasmid. Plates were incubated at 30^{o}C for 2-6 days. (B) Cytoductants from (A) were transformed with a *HIS3*-marked *pCUP1-SUP35NM-GFP* or *pCUP1-RNQ1-GFP* plasmid, patched on selective plates containing 100 μM $CuSO_4$, and incubated in the dark at 30^{o}C before live cell imaging was performed by confocal microscopy. Observations were made of at least three transformants per strain and 100 cells per transformant. Representative images are shown. No variability was seen among cells with respect to the GFP pattern.

3.3.6 Synthetic lethality is due in part to [*PSI*$^+$]-mediated nonsense suppression

To investigate if the lethal effect of [*PSI*$^+$] on *rtf1Δ rkr1Δ* double mutants is due to the presence of a prion or to a reduction in Sup35 function, I introduced the *sup35-Y351C* mutation into our genetic background and asked whether this mutation could inhibit the growth of *rtf1Δ rkr1Δ* [*psi*] double mutants. The *sup35-Y351C* mutation was previously shown to increase read-through of stop codons (BRADLEY *et al.* 2003) and impair the growth of a *rkr1Δ* strain (BENGTSON and JOAZEIRO 2010). Interestingly, *rtf1Δ rkr1Δ sup35-Y351C* [*psi*$^-$] strains grew more slowly than *rtf1Δ rkr1Δ SUP35* [*psi*$^-$] cells (Figure 20). The increased nonsense suppression due to *sup35-Y351C* did not fully recapitulate the effects of [*PSI*$^+$] in *rtf1Δ rkr1Δ* strains; however, this may be due to the effect of the *sup35-Y351C* mutation in our strain background, as [*PSI*$^+$] and *sup35* alleles can cause different phenotypes in different strain backgrounds (TRUE *et al.* 2004). Additionally, my results may indicate that Sup35 aggregates or additional consequences of [*PSI*$^+$], not duplicated by the *sup35-Y351C* allele, contribute to the *rtf1Δ rkr1Δ* genetic interaction. Regardless, the slow growth of *rtf1Δ rkr1Δ sup35-Y351C* cells suggests that an increase in nonsense suppression plays at least a partial role in *rtf1Δ rkr1Δ* [*PSI*$^+$] lethality.

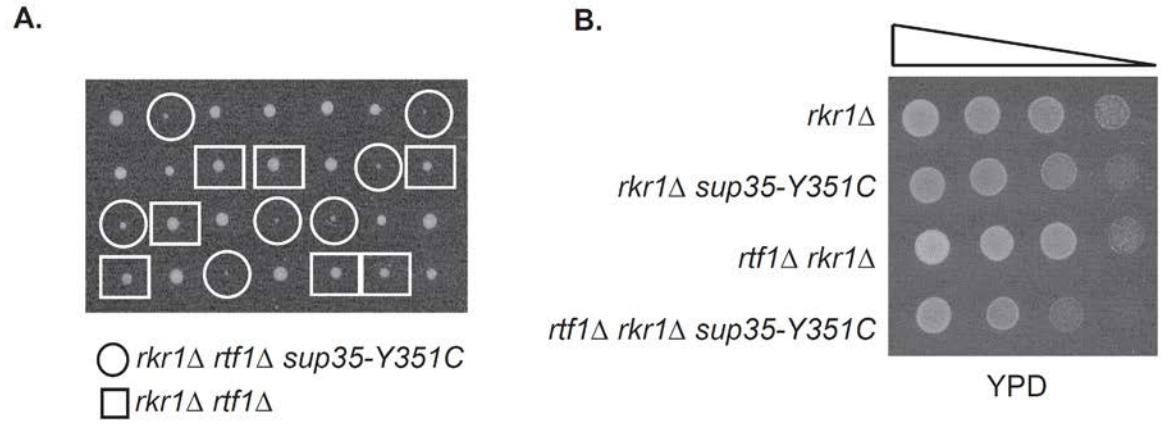

Figure 20: Nonsense suppression impairs growth of *rtf1Δ rkr1Δ* cells

(A) Tetrad dissections of crosses between a [*psi*$^-$] *rkr1Δ sup35-Y351C* strain (KY2292) and a [*psi*$^-$] *rtf1Δ rkr1Δ SUP35* strain (KY2286). Dissections were done on YPD and incubated at 30^{o}C for 2 days**.** Double mutant *rtf1Δ rkr1Δ SUP35* segregants are highlighted by boxes while *rtf1Δ rkr1Δ sup35-Y351C* triple mutants are highlighted by circles. (B) A tetrad from (A) was further analyzed by five-fold serial dilution analysis on YPD and incubated at 30^{o}C for 1 day. Growth differences are less apparent on YPD on later days.

3.3.7 [*PSI*$^+$] impacts *rkr1Δ* phenotypes

My results demonstrate that the presence of [*PSI*$^+$] greatly affects *rkr1Δ* genetic interactions. To further assess the physiological impact of [*PSI*$^+$] on these strains, I assayed the growth of *rtf1Δ* and *rkr1Δ* strains in the presence or absence of prions under conditions of cell stress. Both *rtf1Δ*

and *rkr1Δ* strains are sensitive to caffeine and cadmium chloride (Figure 21A). Interestingly, the $CdCl_2$-sensitivity of *rkr1Δ* strains was strongly alleviated by curing prions through GuHCl treatment (Figure 21A). In addition, a slight ethanol sensitivity of *rkr1Δ* strains was detected only in uncured conditions. Together, these results suggest that prions influence the fitness of strains lacking *RKR1*, particularly under conditions of cell stress. I verified that [*PSI*$^+$] influenced *rkr1Δ* phenotypes by transforming a *rkr1Δ* strain with the *HSP104*, *URE2*, and *LSM4* overexpression plasmids and measuring cadmium chloride sensitivity. Overexpressing these genes suppressed the sensitivity of *rkr1Δ* cells to cadmium chloride, similar to the effects seen in a cured strain obtained by passage on GuHCl (Figure 21A and 21B). Additionally, I tested the effect of the *sup35-Y351C* mutation on the phenotype of a *rkr1Δ* [*psi*$^-$] strain and found that it causes sensitivity to cadmium chloride, as seen for *rkr1Δ* [*PSI*$^+$] cells (Figure 21A and 21C). This result demonstrates that [*PSI*$^+$]-mediated nonsense suppression causes this *rkr1Δ* phenotype and affects the fitness of strains lacking *RKR1*.

While the curing of prions did not suppress the caffeine sensitivity of *rkr1Δ* strains, it did partially suppress the caffeine sensitivity of an *rtf1Δ* strain (Figure 21A). Interestingly, double mutant *rtf1Δ rkr1Δ* strains obtained by dissection onto medium containing GuHCl were more sensitive to caffeine than either cured single mutant strain (Figure 21A). These data demonstrate that *RTF1* and *RKR1* are both required for cell viability under certain growth conditions even in the absence of prions. These results also correlate with our observation that a residual growth defect is apparent for GuHCl-treated *rkr1Δ htb1-K123R* double mutants compared to GuHCl-treated *RKR1 htb1-K123R* strains (Figure 16C).

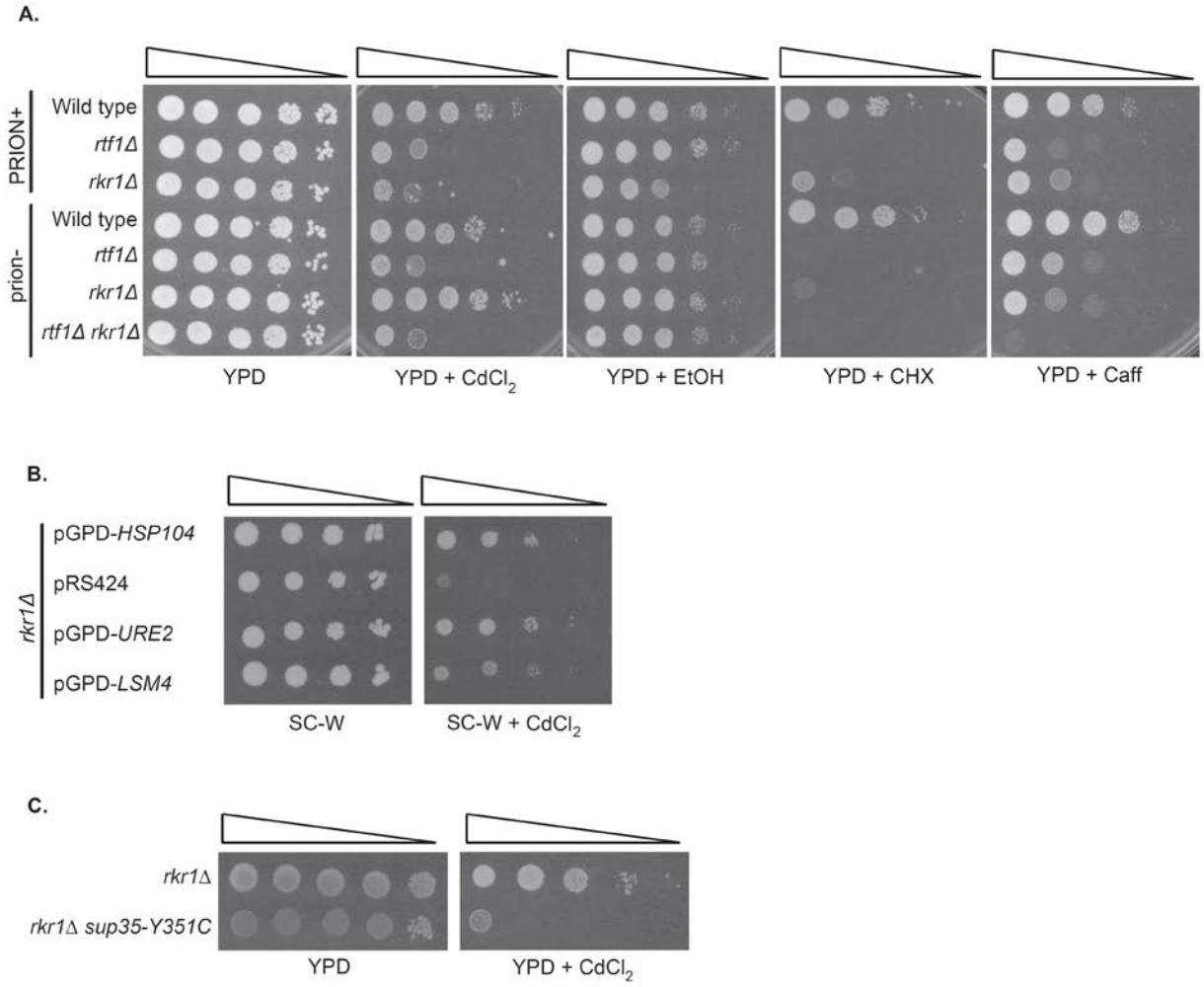

Figure 21: The presence of [*PSI*$^+$] affects *rkr1Δ* phenotypes

(A) Wild type (KY761), *rtf1Δ* (KY2211), and *rkr1Δ* (KY2236) strains, or these strains first passaged onto YPD + 5 mM GuHCl, as well as an *rtf1Δ rkr1Δ* [*psi*$^-$] strain (KY2209) were used for dilution growth assays on YPD or YPD containing 50 µM $CdCl_2$, 10% EtOH, 15 mM caffeine, or 0.8 µg/ml cycloheximide and incubated at 30^{o}C for 2 to 6 days. PRION+ indicates uncured cells; prion- indicates cells passaged on medium containing GuHCl. (B) A *rkr1Δ* strain (KY2236) was transformed with 2µ *TRP1*-marked p*GPD-HSP104*, p*GPD-URE2*, p*GPD-LSM4*, or empty vector (pRS424). Five-fold serial dilution analysis of these transformants was performed on SC-W or SC-W containing 50 µM $CdCl_2$ and incubated 30^{o}C for 3 days. (C) *rkr1Δ* (KY2309) and *rkr1Δ sup35-Y351C* (KY2306) strains were used for ten-fold dilution analysis on YPD and YPD containing 50 µM $CdCl_2$ and incubated at 30^{o}C for 3 days.

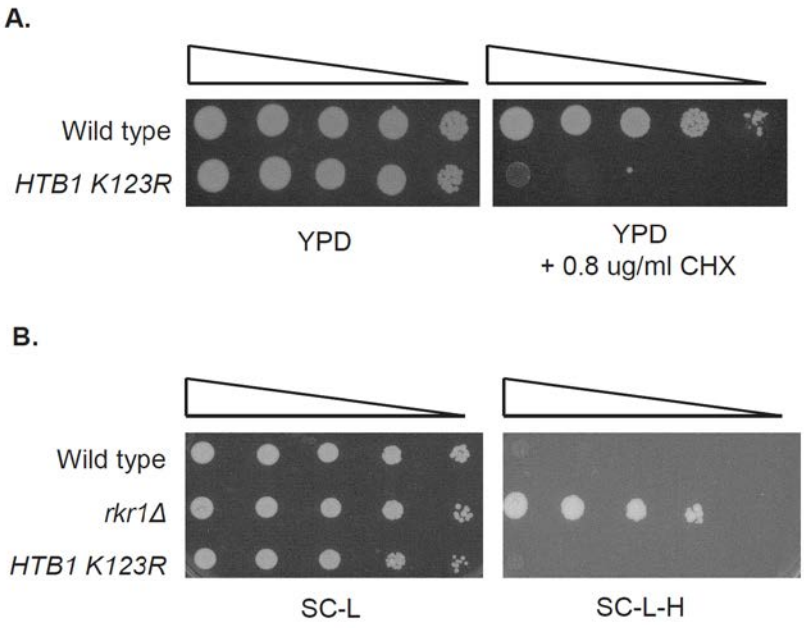

Figure 22: An H2B K123R mutant strain is sensitive to cycloheximide but does not exhibit increased expression of a nonstop reporter.

(A) HTB1 (KY2043) and *htb1-K123R* (KY2044) strains were grown to saturation and diluted to 3 x 10^8 cells/ml. Ten-fold serial dilutions were performed on YPD or YPD containing 0.8 µg/ml cycloheximide (CHX) and incubated at 30^{o}C for 2 or 6 days, respectively. (B) *HTB1* (KY2043) and *htb1-K123R* (KY2044) strains were transformed with a *LEU2*-marked plasmid containing a *his3* nonstop reporter. Strains were then grown to saturation and diluted to 1 x 10^8 cells/ml. Ten-fold serial dilutions were performed on SC-L or SC-L-H and plates were incubated at 30^{o}C for 3 days.

3.3.8 *rtf1Δ* suppresses the elevated levels of nonstop proteins in *rkr1Δ* cells

In my phenotypic analyses, I found that *rkr1Δ* mutants are sensitive to cycloheximide (CHX) (Figure 21A). Furthermore, the *RKR1* genetic interactors, *rtf1Δ* and *htb1-K123R*, also confer sensitivity to CHX (Figure 21A and 22A), and this phenotype is not rescued by clearing cells of prions (Figure 21C). CHX inhibits ribosome translocation during protein synthesis (SCHNEIDER-POETSCH *et al.* 2010) as well as the decay of nonsense and nonstop mRNA (reviewed in(WAGNER and LYKKE-ANDERSEN 2002)) (FRISCHMEYER *et al.* 2002). Therefore, the CHX-sensitivity of cells lacking Rkr1 or Rtf1 may indicate a requirement for these proteins under conditions of impaired translation and/or mRNA quality control.

In previous studies, cells lacking Rkr1 exhibited an increase in nonstop protein levels without affecting the levels of nonstop mRNA (BENGTSON and JOAZEIRO 2010; WILSON *et al.* 2007). As the prion form of Sup35, [*PSI*$^+$] results in suppression of nonsense codons in nonsense mRNAs as well as read-through of normal stop codons (PAUSHKIN *et al.* 1996; WILSON *et al.* 2005). Rkr1 has been reported to interact with ribosomes and be localized in the cytoplasm, where it is then necessary for nonstop protein degradation (BENGTSON and JOAZEIRO 2010; FLEISCHER *et al.* 2006). Because our previous studies indicated nuclear localization of Rkr1 (BRAUN *et al.* 2007), I decided to re-examine the localization of Rkr1 using different strains, an N-terminally tagged HA-Rkr1 construct, and better visualization using confocal microscopy. Here, I found that Rkr1 is predominantly, though not exclusively, cytoplasmic, thus supporting its role in nonstop protein degradation in our strains (Figure 23).

Given the role of Rkr1 in degrading nonstop proteins and the role of [*PSI*$^+$] in generating nonstop proteins, I hypothesized that the lethality of *rtf1Δ rkr1Δ* [*PSI*$^+$] cells might be due to an overabundance of these proteins. To test this idea and examine if *RTF1* plays a role in regulating

nonstop protein levels, I used a nonstop reporter plasmid containing the *HIS3* gene without a stop codon (WILSON *et al.* 2007). Because wild type cells carrying the reporter efficiently degrade the *his3* nonstop transcript and protein, they fail to grow on media lacking histidine (WILSON *et al.* 2007). However, cells lacking *RKR1* are unable to degrade the His3 nonstop protein, and therefore grow on media lacking histidine (Figure 24A) (WILSON *et al.* 2007). Cells lacking *RTF1* alone did not exhibit a *his3* nonstop phenotype in the presence or absence of prions (Figure 24A). Interestingly, however, absence of the histone H2A variant Htz1, which was previously shown to increase nonstop transcript levels (WILSON *et al.* 2007), only exhibited a *his3* nonstop phenotype in [*PSI*$^{+}$] conditions (Figure 24A). This result indicates that Sup35 aggregation exacerbates the nonstop phenotype in some strains, though not detectably in *rkr1Δ* strains by this assay. As for *rtf1Δ* cells, I did not observe a *his3* nonstop phenotype for H2B K123R strains, suggesting that loss of H2B ubiquitylation does not cause an increase in nonstop RNA or protein levels as measured by this reporter (Figure 22B).

To test if deletion of *RTF1* and *RKR1* additively elevate nonstop protein levels, we measured expression of the *his3* nonstop reporter in viable, cured *rtf1Δ rkr1Δ* strains. Surprisingly, deletion of *RTF1* suppressed the *his3* nonstop phenotype of a *rkr1Δ* mutant, as indicated by reduced growth on –His medium (Figure 24B). I confirmed this result using a second nonstop reporter in which the Protein A gene lacks a stop codon (WILSON *et al.* 2007). Total Protein A levels were measured by western analysis (Figure 24C). Using this assay, I observed that the curing of prions caused a decrease in nonstop Protein A levels in the *rkr1Δ* strain (Figure 24C). Also, in agreement with results obtained with the *his3* nonstop reporter, *rtf1Δ rkr1Δ* double mutants had reduced nonstop Protein A levels compared to *rkr1Δ* cells

(Figure 24C). These results argue against a simple model in which a combinatorial increase in nonstop protein levels causes *rtf1Δ rkr1Δ* synthetic growth defects.

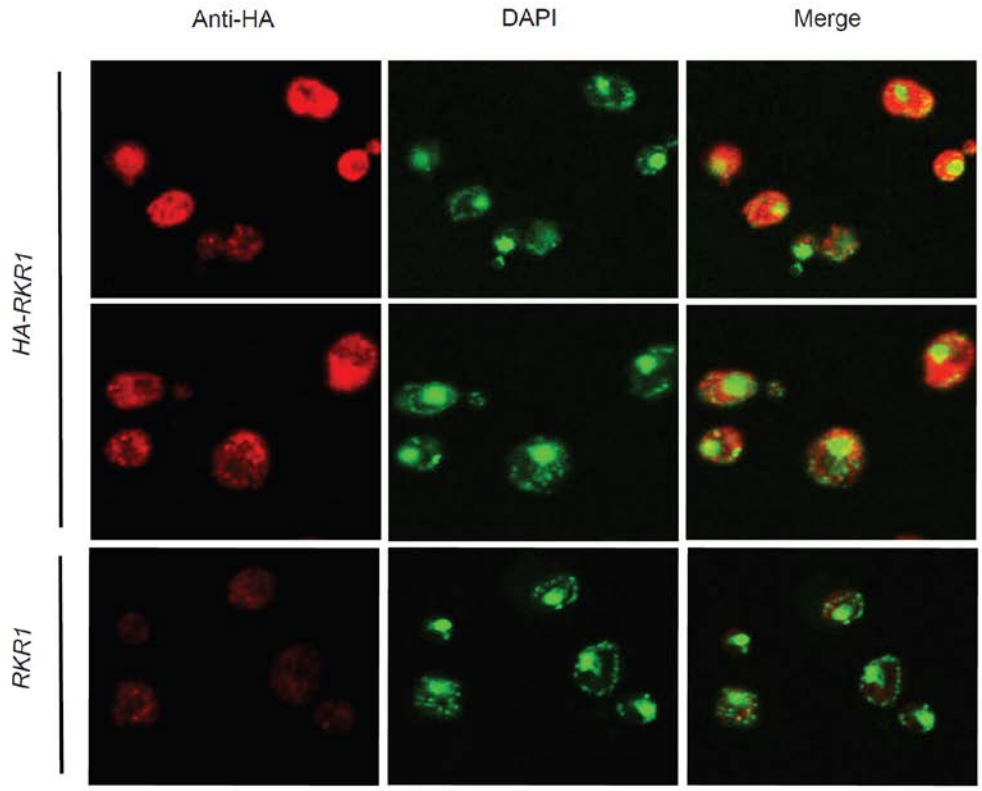

Figure 23: Localization of HA-Rkr1 is predominantly cytoplasmic.

(KY2289) was transformed with either an H*A-RKR1* (pMB11) or untagged *RKR1* (pPC65) plasmid and grown to mid log phase before fixation and incubation with anti-HA antibody primary (Roche) and Alexa Red 647 secondary (Molecular Probes). Cells were mounted with ProLong DAPI stain (Invitrogen) and visualized using confocal microscopy.

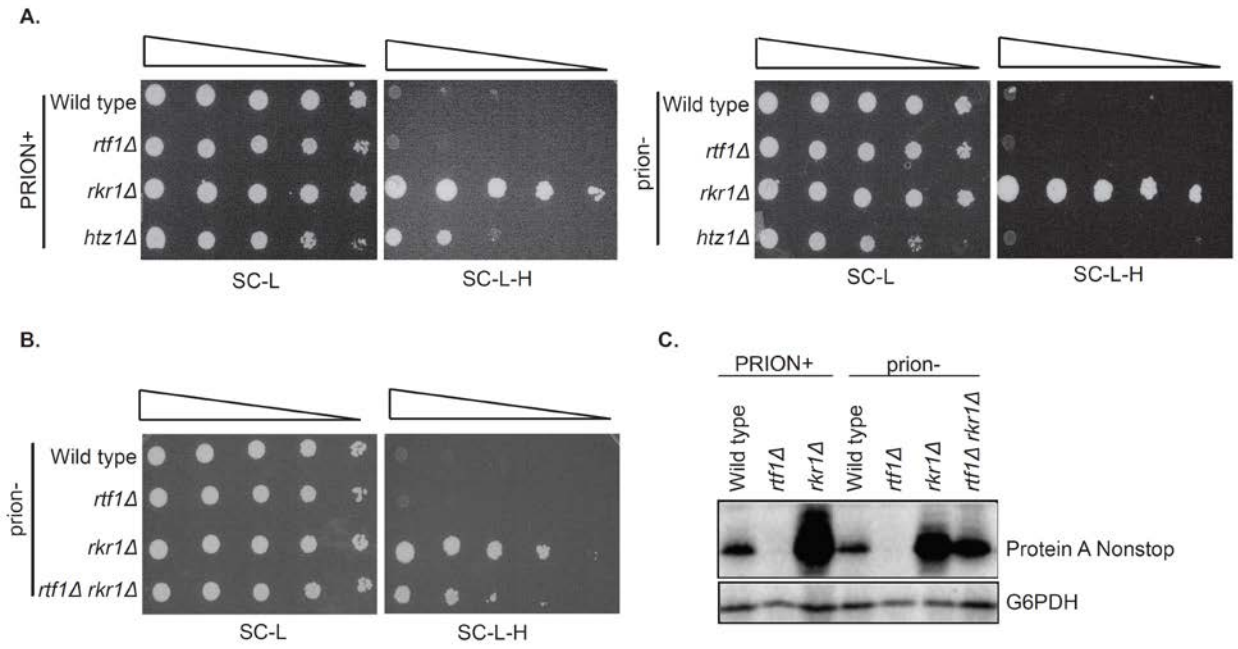

Figure 24: *rtf1Δ rkr1Δ* strains exhibit a decrease in nonstop reporter proteins

(A and B) WT (KY1030), *rtf1Δ* (KY564), *rkr1Δ* (KY2202), *rtf1Δ rkr1Δ* (KY2210), and *htz1Δ* (KY1404) strains were transformed with a *LEU2*-marked plasmid containing a *his3* nonstop reporter. Ten-fold serial dilutions were plated on SC-L or SC-L-H medium and incubated at 30^{o}C for 2 or 3 days, respectively. prion- strains were generated prior to transformation by curing on medium containing 2.5 or 5 mM GuHCl. (C) WT (KY307), *rtf1Δ* (KY2211), *rkr1Δ* (KY2236), and *rtf1Δ rkr1Δ* (KY2209) were transformed with a *URA3*-marked plasmid containing a protein A nonstop reporter and grown to early log phase in SC-U containing 2% galactose. prion- strains were generated as in (A). TCA extracts were analyzed by western blotting using antibodies against Protein A or G6PDH, which served as a loading control. PRION+ indicates uncured cells; prion- indicates passage on medium containing GuHCl.

3.4 DISCUSSION

I investigated the genetic relationship between the Paf1c subunit Rtf1 and the ubiquitin-protein ligase Rkr1 to better understand the interaction between their transcription and protein quality control functions. A transposon mutagenesis screen for genetic suppressors of *rtf1Δ rkr1Δ* synthetic lethality identified a mutation in the gene encoding Hsp104. Further investigation into the suppression mechanism of an *hsp104* mutation showed that *rtf1Δ rkr1Δ* strains are inviable only in the presence of [*PSI*$^+$]. The overexpression, deletion, or GuHCl-mediated inactivation of *HSP104*, as well as the overexpression of the prion-coding genes *URE2* and *LSM4*, all rescue *rtf1Δ rkr1Δ* synthetic lethality and clear cells of [*PSI*$^+$]. [*PSI*$^+$], the prion aggregate of Sup35, a translation termination factor necessary for proper stop codon recognition, results in read through of normal stop codons (PAUSHKIN *et al.* 1996; WILSON *et al.* 2005). In turn, Rkr1 is required for the efficient ubiquitylation and degradation of nonstop proteins by recognition of a poly-lysine tract resulting from translation through the poly(A) tail (BENGTSON and JOAZEIRO 2010). I have shown that [*PSI*$^+$] and resulting nonsense suppression conditions exacerbate *rkr1Δ* phenotypes, suggesting that the presence of [*PSI*$^+$] and excess nonstop proteins is detrimental in the absence of *RKR1*. However, it is only in the absence of Rtf1 that this increased burden on protein quality control machinery causes inviability.

Improper recognition of stop codons leads to read-through of both normal and premature stop codons, resulting in nonstop proteins and nonsense suppression, respectively, and explaining the multiple phenotypic effects of [*PSI*$^+$] (TRUE *et al.* 2004; WILSON *et al.* 2005). In addition, because degradation of many aberrant mRNAs depends on proper translation termination, the presence of [*PSI*$^+$] also affects the degradation of these transcripts (WILSON *et al.* 2005).

Therefore, in combination, the presence of [*PSI*$^+$] and the absence of *RKR1* likely results in increased levels of nonstop proteins that cannot be efficiently recognized and degraded, as well as an increased burden on mRNA quality control. Though I have shown that [*PSI*$^+$] influences the phenotypic consequences of deleting *RKR1* in yeast, added stress to protein or mRNA quality control systems could also impact the severity of *rkr1* mutations in higher eukaryotes and contribute to the development of diseases, such as neurodegeneration (CHU *et al.* 2009).

In addition to mechanisms that recognize and degrade aberrant proteins, such as that involving Rkr1, several mRNA surveillance pathways also prevent the translation of erroneous transcripts. Two such quality control pathways are nonsense-mediated decay, which recognizes transcripts with premature termination codons, and nonstop decay, which recognizes transcripts without stop codons (reviewed in (FASKEN and CORBETT 2005)). Cells deficient in Paf1c exhibit transcript-specific increases or decreases in mRNAs targeted for quality control, presumably due to errors in RNA processing (PENHEITER *et al.* 2005; STRAWN *et al.* 2009). For example, mutations in yeast *PAF1* result in shortened poly(A) tails as well as altered poly(A) site utilization, which produce substrates for mRNA surveillance pathways (MUELLER *et al.* 2004; NORDICK *et al.* 2008a; PENHEITER *et al.* 2005), and defects in hPaf1c give rise to aberrant transcripts, which are inefficiently processed or exported (FARBER *et al.* 2010; NAGAIKE *et al.* 2011). In agreement with these earlier observations, I have shown that deletion of *RTF1* results in decreased protein product from two nonstop reporters (Figure 24), presumably because the absence of Rtf1-dependent histone modifications leads to transcriptional alterations and/or effects on RNA export, stability, or translation.

The mechanism by which Rtf1 and H2B ubiquitylation protects cells against the combined lethal effects of [*PSI*$^+$] and *rkr1*Δ is not clear. My initial hypothesis was that deletion

of *RTF1* elevates nonstop protein synthesis or stability to levels that are intolerable in [*PSI*$^+$] *rkr1*Δ strains; however my nonstop reporter assays detected decreased, not increased, nonstop protein levels in *rtf1*Δ strains. Therefore, the synthetic lethality between *rtf1*Δ and *rkr1*Δ is not easily explained by an elevation in nonstop protein levels. An alternative explanation, based on the importance of Paf1c in transcription and RNA processing (reviewed in (CRISUCCI and ARNDT 2011b; JAEHNING 2010)), is that *rtf1*Δ leads to a spectrum of aberrant transcripts that may impose stress on the cell or impair the expression of specific genes whose products play a role in RNA surveillance or protein quality control. These products would be especially important in cells lacking Rkr1 and containing [*PSI*$^+$]. My observation that *rtf1*Δ and *rkr1*Δ mutants have similar stress-related phenotypes (Figure 22) further supports a role for both proteins in preventing the accumulation of quality control substrates. To address this hypothesis, RNA-seq could be used to identify differences in mRNA in wild type compared to *rtf1Δ* cells. Finally, my data do not preclude the possibility that Rkr1 possesses alternate activities, which remain to be identified, and it is the absence of these functions that elevates the need for Paf1c and its associated histone modifications. Regardless of the precise mechanism, my results reveal a previously unrecognized requirement for a functional Paf1c and its associated histone modifications in protecting cells from the adverse effects of [*PSI*$^+$] in the context of impaired protein quality control.

Table 3: *S. cerevisiae* strains used in Chapter 3

Strain	Genotype
KY307	*MATα his3Δ200 ura3-52 trp1Δ63 lys2Δ202*
KY453	*MATα rtf1Δ100::URA3 his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-173R2*
KY564	*MATα rtf1Δ102::ARG4 his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-173R2 arg4-12*
KY607	*MATα rtf1Δ101::LEU2 his3Δ200 leu2Δ1 ura3-52 lys2-128δ*
KY761	*MATα his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY958	*MATα rtf1Δ101:LEU2: his3Δ200 leu2Δ1 ura3Δ0 trp1Δ63 lys2-128δ*
KY1030	*MATα his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY1404	*MATα htz1Δ::KanMX his3Δ200 leu2Δ0 ura3Δ0 lys2Δ0*
KY1663	*MATα rtf1Δ102::ARG4 rkr1Δ::KanMX leu2Δ1 ura3-52 arg4-12 [pKA69: RTF1/URA3/C/A]*
KY2043	*MAT**a** hta2-htb2Δ::KanMX his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY2044	*MAT**a** HTA1-htb1K123R hta2-htb2Δ::KanMX his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY2202	*MAT**a** rkr1Δ::HIS3 his3Δ200 leu2Δ1 ura3-52*
KY2203	*MAT**a** hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 his3Δ200 leu2Δ1 ura3-52 trp1Δ63* [*URA3/HTA1-HTB1/C/A*] [*HIS3/HTA1-FLAG-htb1-K123R/C/A*]
KY2204	*MAT**a** hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 rkr1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 trp1Δ63* [*URA3/HTA1-HTB1/C/A*] [*HIS3/HTA1-FLAG-htb1-K123R/C/A*]
KY2205	*MAT**a** rtf1Δ102::ARG4 rkr1Δ::KanMX leu2Δ1 ura3-52 arg4-12 trp1Δ63 [pKA69: RTF1/URA3/C/A]*
KY2209	*MATα rtf1Δ::KanMX rkr1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 trp1Δ63* [*psi*$^-$]
KY2210	*MAT**a** rtf1Δ::KanMX rkr1Δ::KanMX his3Δ200 leu2Δ1 trp1Δ63* [*psi*$^-$]
KY2211	*MATα rtf1Δ::KanMX his3Δ200 leu2Δ1 ura3Δ0 trp1Δ63*
KY2236	*MATα rkr1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY2286	*MATα rtf1Δ101::LEU2 rkr1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 ade1-14* [*psi*$^-$]
KY2289	*MAT**a** rkr1Δ::HIS3 his3Δ200 leu2Δ1 trp1Δ63 lys2-128δ*
KY2292	*MAT**a** rkr1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 ade1-14* [*psi*$^-$]
KY2306	*MAT**a** rkr1Δ::KanMX sup35-Y351C his3Δ200 leu2Δ1 ura3-52 trp1Δ63 ade1-14* [*psi*$^-$]
KY2309	*MAT**a** rkr1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 ade1-14* [*psi*$^-$]
L2261	*MAT**a** kar1 ura2 leu2 his* [*PIN*$^+$]
L2265	*MAT**a** kar1 ura2 leu2 his* [*PSI*$^+$]

4.0 INVESTIGATIONS INTO RKR1 SUBSTRATES AND INTERACTIONS

4.1 INTRODUCTION

Numerous proteins are targeted for ubiquitylation in the cell, often as a prerequisite to degradation, but also as a part of a signaling event. The conserved 76 amino acid ubiquitin protein is conjugated to lysine resides on its target protein by a chain of ubiquitylation components. In yeast, this pathway consists of one essential E1 ubiquitin activating enzyme, Uba1, which links to ubiquitin through a high-energy bond. Ubiquitin is then transferred to one of 11 E2s, or ubiquitin conjugating enzymes. An E2 then catalyzes ubiquitylation of a substrate with the assistance of one of hundreds of E3s, or ubiquitin ligases, which aid in substrate specificity (reviewed in (HOCHSTRASSER *et al.* 1999)). When targeted for degradation by this pathway, a chain of ubiquitin is added to the substrate one at a time in a rapid and repeated sequence of events that makes these interactions difficult to capture, after which the protein is then degraded by the 26S proteasome (reviewed in (DESHAIES and JOAZEIRO 2009; HOCHSTRASSER *et al.* 1999)).

Global analysis studies have been conducted in several organisms to uncover protein-protein interactions in the ubiquitin pathway (KAISER and HUANG 2005). However, given the transient nature of interactions necessary to degrade a substrate, these high-throughput studies are difficult. To identify E2-E3 interactions in humans, one group used a yeast-two hybrid screen

with the catalytic domains of over 30 human E2s and 250 E3 domains, confirming several known interactions and finding over 300 potential interactions (VAN WIJK *et al.* 2009). Another proteomic study isolated all ubiquitylated proteins in yeast and subjected them to mass spectrometry, identifying more than 1000 different proteins that are ubiquitylated at any given time (PENG *et al.* 2003). Deciphering the factors that are involved in the ubiquitylation of each of these proteins is a more arduous task. A similar method of ubiquitin profiling isolated all ubiquitylated proteins in wild type yeast and in yeast cells lacking the proteasomal ubiquitin receptor Rpn10 to identify substrates dependent on Rpn10 for degradation (MAYOR *et al.* 2005). Another study involving yeast used a screened of 188 purified potential substrates for ubiquitylation by the E3 Rsp5 using a novel luminescent assay *in vitro* (KUS *et al.* 2005). The same group used commercially available protein microarrays to screen for additional Rsp5 candidate substrates (GUPTA *et al.* 2007). However, this assay relied on the ability to optimize conditions using a known Rsp5 substrate, a piece of data which is not known for many of the hundreds of ubiquitin ligases in the cell. In addition, while these global analyses are helpful in identifying potential interactions, the next challenge is to verify these substrates, many of which are targeted for degradation, with *in vivo* and *in vitro* assays (BLOOM and PAGANO 2005).

RKR1 was originally identified in a screen for mutations that were synthetically lethal in the absence of Rtf1 of the Paf1 complex in yeast (BRAUN *et al.* 2007). *RKR1* encodes a protein with a conserved N-terminal region and a RING-finger domain, which possesses *in vitro* ubiquitin ligase activity (BRAUN *et al.* 2007). The isolated mutation that conferred lethality in the context of *rtf1Δ* contained an early stop codon that truncated the protein before this RING domain. RING (Really Interesting New Gene) domain proteins represent a class of ubiquitin ligases of which there are hundreds in yeast and humans (reviewed in (DESHAIES and JOAZEIRO

2009)). In addition, structural analyses of E2-E3 complexes have shown that this RING domain is an important component of ubiquitin ligase-ubiquitin conjugase binding and activation, indicating its essential nature in this function (DOMINGUEZ *et al.* 2004; ZHENG *et al.* 2000). RING E3s thus direct transfer of ubiquitin from an E2 to the substrate, an activity that is mechanistically different from that of HECT domain E3s, which are able to form an intermediate with ubiquitin to directly transfer it to its substrate (reviewed in (DESHAIES and JOAZEIRO 2009)). Therefore while the conserved RING domain of Rkr1 is most likely important for its E2 interaction, the conserved N-terminal region of Rkr1 may be involved with substrate specificity.

Rkr1 is predominantly cytoplasmic, localizes with ribosomes, and has been implicated in protein quality control of nonstop proteins (BENGTSON and JOAZEIRO 2010; WILSON *et al.* 2007). At the start of this project, none of this information was known and possible substrates and interacting partners for Rkr1 were of high interest to furthering our understanding of this protein and its requirement in the absence of *RTF1*. In this chapter, I will discuss how I used a wide range of techniques to find factors that interact with Rkr1 or require Rkr1 for ubiquitylation in yeast, including phenotypic studies, genetics, cross-linking and immunoprecipitation of Rkr1, as well as targeted studies of potential substrates.

4.2 MATERIALS AND METHODS

4.2.1 Yeast strains and growth conditions

KY *Saccharomyces cerevisiae* yeast strains are isogenic with FY2, a *GAL2*$^{+}$ derivative of S288C (WINSTON *et al.* 1995). Yeast deletion mutants, crosses, and transformants were created using

standard protocols (AUSUBEL 1988; ROSE 1991). Yeast were grown on rich (YPD), synthetic complete (SC) or synthetic minimal (SD) as specified and prepared as previously described (ROSE 1991). Media to test growth phenotypes were was prepared as described in the figure legends and used for 10-fold dilution analysis with the indicated number of cells.

4.2.2 His-Ubiquitin Assays

This assay was performed as previously described (MURATANI *et al.* 2005). Briefly, strains were transformed with plasmids expressing potential substrates (described in figure legend) and HIS-tagged or untagged ubiquitin expressed from a copper-inducible promoter. Strains were induced during early log phase for four hours with 500 µM $CuSO_4$, collected, and lysed in a denaturing 6M guanidine-hydrochloride buffer. An equal amount of lysate (2-5 mg) was incubated with Ni-NTA beads (Qiagen) and rotated 2 hours at RT before washing and eluting by boiling in 2X Sample buffer + 0.2 M imidazole and running on a 7-20% gradient gel, transferred with nitrocellulose, and analyzed by western analysis with anti-HIS for efficiency of immunoprecipitation and antibody against the tagged substrate to detect it in the immunoprecipitated sample.

4.2.3 Proteasome inhibition and detection of ubiquitylation

Strains were grown in the presence of SC+0.1% proline overnight and diluted to an OD_{600} of 0.5 in fresh SC+ 0.1% proline + 0.003% SDS to allow transient permeabilization of the cell membrane as previously described (LIU *et al.* 2007). Cells were grown at 30^{o}C for 3 hours before addition of MG132 (75 µM final; Sigma) or equivalent microliters of DMSO (Sigma) and grown

for another 30 minutes. Cells were collected and lysates were made using traditional bead beating methods with Buffer Ub (40 mM HEPES-NaOH pH7.5, 350 mM NaCl, 0.1% Tween-20, 10% glycerol, 20mM NEM, 1X Protease inhibitors). Immunoprecipitations were done with 3 mg of protein added to anti-FLAG M2 resin (Kodak) and rotated for 2 hours at 4^{o}C before loading on a 7-20% gradient gel, transferred to PVDF, and analyzed by western analysis.

4.2.4 Cross-linking and immunoprecipitation

Strains were grown to early log phase in SC-Trp media to select for plasmids that express *RKR1* (pPC65) or modTAP-RKR1 (pMB67), which harbors a modified TAP tag containing a FLAG-protein A sequence. Cross-linking was performed using 1% formaldehyde for the indicated length of time before being quenched with a G/T buffer (3M Glycine, 20 mM Tris). Extracts were made using traditional bead beating methods in 1 mL SUME buffer (10 mM MOPS pH6.8, 1% SDS, 10 mM EDTA, 1X Protease Inhibitors). For immunoprecipitation, total lysate was incubated with 100 µl IgG agarose (Sigma) for 2 hours and washed (50 mM NaCl, 10 mM Tris) and resuspended in 80 µl SUME + 0.005% Bromophenol Blue. After boiling at 100^{o}C for 3', samples were loaded onto two 8% SDS POLYACRYLAMIDE gels. One (5 µl) was used for western analysis to confirm immunoprecipitation. The remainder was run 1 cm into the running gel and the gel slices were excised, destained, and sent for sequencing in collaboration with Dr. Richard Gardner at the University of Washington.

4.2.5 Western analysis

Western analysis was performed by standard methods using nitrocellulose membranes unless otherwise described. Blots probed with anti-ubiquitin antibody were first boiled in water for 5' prior to blocking with 5% milk. Antibodies used include Anti-ubiquitin (1:50 Santa; Cruz). anti-FLAG M2 (1:1000; Sigma), anti-His (1:1000; GE Healthcare), and anti-G6PDH (1:50,000; Sigma). Immunoreactivity was measured using chemiluminescence (Perkin-Elmer) and a 440 CF digital imaging station (Kodak).

4.3 RESULTS

4.3.1 The RING domain of Rkr1 is required for resistance to cycloheximide

To better understand the function of Rkr1 in yeast, I performed a screen for phenotypes affected by the deletion or overexpression of *RKR1*. These phenotypes covered a wide range of processes, including cell division and signaling as well as undefined phenotypes. In yeast, these data can help to classify and categorize a gene, which can functionally connect it to other genes. Prior to this screen, Mary Braun demonstrated that *rkr1Δ* strains are sensitive to media lacking inositol, a phenotype generally connected to errors in transcription which is also seen in strains lacking members of the Paf1 complex (BRAUN *et al.* 2007).

Interestingly, from my extensive screen only one new phenotype for Rkr1 emerged. *rkr1Δ* strains are sensitive to media containing low levels of cycloheximide (Figure 25). I was

unable to complement this phenotype with a plasmid containing *RKR1*, which could indicate a secondary mutation is the cause of this phenotype or that a precise level of Rkr1 is necessary for cycloheximide resistance. However, I did observe this phenotype in multiple *rkr1Δ* strains (Figure 25 and data not shown). I also performed tetrad analysis of a *rkr1Δ/RKR1* heterozygous diploid and showed that this phenotype sorted 2:2 with the absence of *RKR1* (Data not shown). The original *rkr1* isolate that is synthetically lethal with *rtf1* strains contains a premature termination codon before the RING domain (Δ RING; Figure 25). The Rkr1 protein from this strain is expressed at levels similar to wild type (Mary Braun, unpublished data). This mutation also causes cycloheximide sensitivity, indicating that cycloheximide resistance is dependent on the RING domain of Rkr1 (Figure 25).

Though the reason for this phenotype is not entirely clear, there is a connection between cycloheximide and aberrant mRNAs that is described in Chapter 3 of this thesis. Alternatively, cycloheximide sensitivity could indicate ubiquitin depletion in *rkr1Δ* strains (HANNA *et al.* 2003). However, I have not observed a decrease in total ubiquitin in any experiment with *rkr1Δ* cellular lysates (data not shown). Interestingly, I have also shown in Chapter 3 that deletion of *RTF1* and substitution of the H2B ubiquitylation site results in cycloheximide sensitivity, indicating that this phenotype is paralleled in *rkr1Δ* genetic interactors. However, deletion of *SAN1*, a RING finger ubiquitin ligase involved in quality control (GARDNER *et al.* 2005), does not exhibit this phenotype (data not shown). This suggests that this cycloheximide phenotype is not a general property of strains lacking quality control ubiquitin ligases. Although this phenotype does not define a function for Rkr1, I reasoned it could still help me identify other proteins that worked with Rkr1 (See Appendix for additional experiments). Therefore, using the

cycloheximide and inositol phenotypes as well as others, I next sought to identify additional players in a pathway with Rkr1.

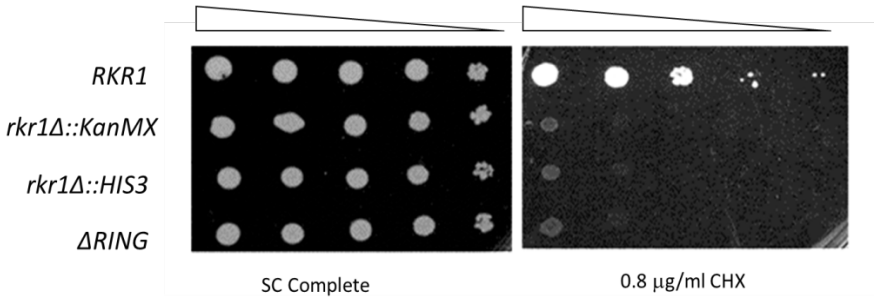

Figure 25: The RING domain of Rkr1 is required for cycloheximide (CHX) resistance.

Wild type (KY1198), *rkr1Δ::KanMX* (MBY29), *rkr1Δ::HIS3* (MBY6B), and *rkr1*Δ RING (SHY17) strains were grown to saturation and used in a 10-fold serial dilution analysis starting with $2x10^8$ cells/ml on SC Complete or SC complete containing 0.8 µg/ml CHX. Plates were incubated 2 (left) or 6 days (right) at 30^oC. Both MBY29 and MBY6B contain clean deletions of *RKR1* replaced by either a *KanMX* or *HIS3* cassette. SHY17 was originally identified as the SL505 mutation, uncovered by Patrick Costa's screen for strains that were synthetically lethal with *rtf1Δ*.

4.3.2 Phenotypes and genetics of *rkr1Δ* interactions

I hypothesized that the loss of genes that encode proteins which physically interact with Rkr1 or are required for its function, such as a ubiquitin conjugase (E2), would exhibit a similar genotypic and phenotypic profile to the loss of *RKR1*. Therefore, using the known phenotypes

and genetics of Rkr1 described above and in Chapter 3, I first screened mutations in E2 genes for a similar pattern of phenotypes (Figure 26 and 27). To this end, I used several E2 mutations that I made or obtained in our strain background, as well as several from the yeast deletion collection (Table 4). There are 11 E2s in *S. cerevisiae*, two of which are essential for viability. Anthony Piro introduced a temperature sensitive *cdc34-2* allele in our background which I was able to use for these experiments. *UBC1* is the second essential E2. However, at the time of these studies, there was no conditional allele of *UBC1* to test for *rkr1Δ* -associated phenotypes and therefore it is not represented in my experiments.

I screened these E2 mutant strains using serial dilution analysis for their ability to grow on medium containing cycloheximide, cadmium chloride, or caffeine, or on medium lacking inositol (Figure 26 and 27). All but 3 E2 mutations tested were sensitive to medium containing caffeine, suggesting this is a more general phenotype of these gene mutations and may not be useful in identifying an E2 partner for Rkr1. Because the loss of *RKR1* results in accumulation in *his3* nonstop protein from a plasmid reporter, I also transformed these strains and assayed for a *his3* nonstop phenotype (Table 4 and Chapters 2, 3) (WILSON *et al.* 2007). While none of the mutations tested caused a *his3* nonstop phenotype, several E2 mutations did reveal other similarities to loss of *RKR1* (Summarized in Table 4). For example, a *rad6Δ* mutation paralleled a *rkr1Δ* strain in all other phenotypes (Figures 26 and 27, Table 4). However, because Rad6 is required for H2B-K123 ubiquitylation that is dependent on Rtf1, these phenotypes may also be mirroring this pathway (HWANG *et al.* 2003). In addition to *rad6Δ* strains, the *cdc34-2* and *ubc4Δ* strains also showed similar, though not identical, phenotypes to loss of *RKR1* or *RTF1* (Figures 26 and 27, Table 4). It is also important to note that loss of Rkr1 causes cadmium chloride sensitivity in our yeast background, but not of a *rkr1Δ* strain from the deletion collection (Figure

27). This may be due to a simple difference in yeast strain background. However, I showed in Chapter 3 that *rkr1Δ* strain are sensitive to medium containing cadmium chloride only in the presence of the prion [*PSI*$^{+}$]. Therefore, this result may also indicate that the deletion collection strains do not contain a high level of this prion, and therefore this phenotype may not be useful with these strains.

Because *rtf1Δ rkr1Δ* strains are inviable, I next hypothesized that loss of a different factor in the same pathway as Rkr1 would display the same genetics. To this end, an E2 mutation combined with loss of *RTF1* should also be lethal. Similarly, because they are in the same pathway, the combination of this E2 mutation and loss of *RKR1* would most likely not have a genetic interaction (Figure 4). To address this hypothesis, I performed tetrad analysis between *rkr1Δ* or *rtf1Δ* strains with *rad6Δ*, *cdc34-2*, *ubc4Δ,* or *ubc5Δ* strains. However, no genetic interactions were observed in any of these crosses (Summarized in Table 4).

A study of human E2 and RING E3 interactions using a yeast two-hybrid screen identified hundreds of potential interactions between E2-E3 partners. Interestingly, this study found that human Ubc4 and Ubc5 physically interacted with the RING domain of human Rkr1 (VAN WIJK *et al.* 2009). Ubc4 and Ubc5 are considered at least partially redundant E2s within yeast, and the double mutation of these genes is either very sick or lethal (SEUFERT and JENTSCH 1990; STOLL *et al.* 2011). While loss of *UBC4* exhibited some phenotypes similar to loss of *RKR1*, the deletion of *UBC5* was not identical in phenotypes to the deletion of *UBC4*, suggesting these two genes have some non-overlapping roles in yeast (Figure 26, 27 and Table 4). Because both strains initially used in my analysis were obtained from the yeast deletion collection and hence are different strain background which may have different phenotypic consequences, I next introduced a *ubc4Δ* mutation in our strain background to perform additional studies. Again, I

expected the loss of the E2 partner for Rkr1 to genetically mimic the loss of *RKR1*. However, I did not find that deletion of *ubc4Δ* caused sickness in the absence of *RTF1* (data not shown). Therefore, while a physical interaction may still exist between Ubc4 and Rkr1, or even between Rkr1 and Ubc5, Rad6, or Cdc34, my data on E2-Rkr1 interactions in yeast would suggest that Rkr1 may have multiple partners in the cell necessary for its function.

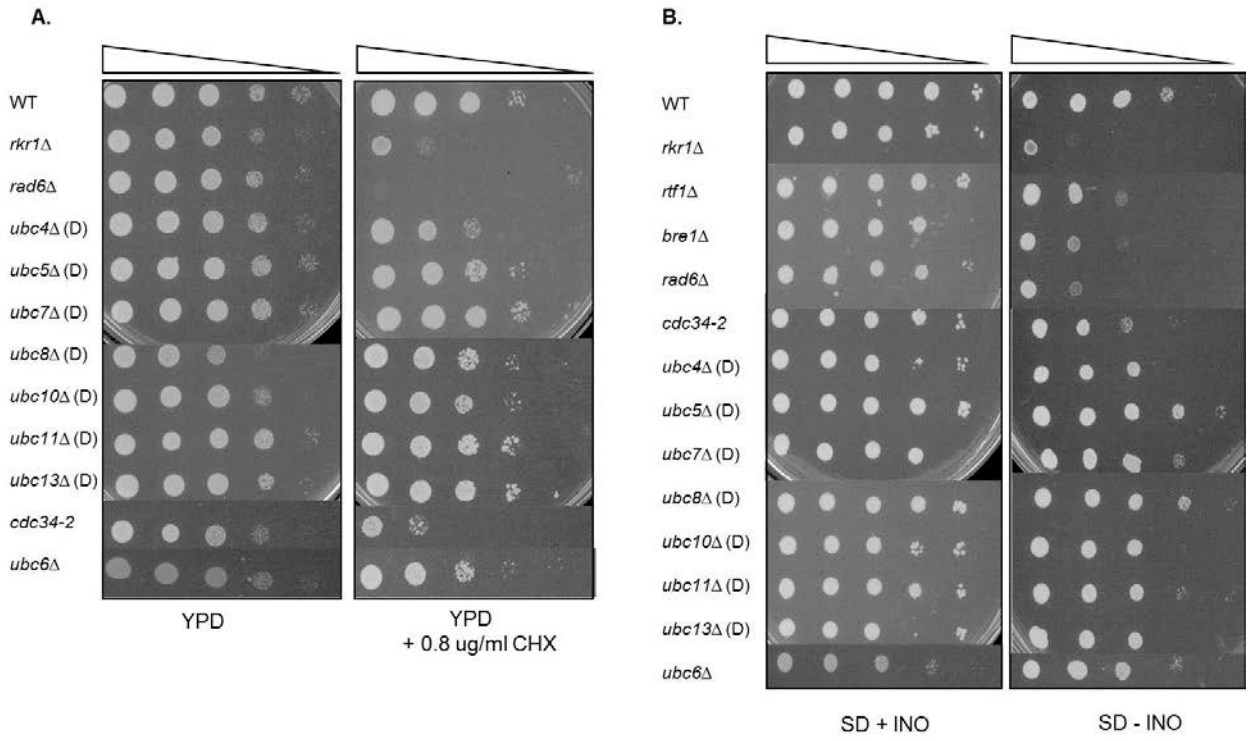

Figure 26: Some E2 mutants display inositol or cycloheximide sensitivity phenotypes.

Strains obtained from the yeast deletion collection are denoted as (D). (A) Wild type (OY8), *rad6Δ* (KY931), *rkr1Δ* (MBY36), *bre1Δ* (KY961), *rtf1Δ* (ECY1), *cdc34-2* (KY1363), *ubc4Δ* (OKA196), *ubc5Δ* (OKA197), *ubc6Δ* (KY1606), *ubc7Δ* (OKA201), *ubc8Δ* (OKA198), *ubc10Δ* (OKA125), *ubc11Δ* (OKA199), and *ubc13Δ* (OKA200) strains were grown to saturation and used in a 10-fold dilution analysis starting with $1x10^8$ cell/ml on SD or SD – inositol. Plates were incubated 2 or 3 days at 30°C. (B) Wild type (OY8), *rad6Δ* (KY931), *rkr1Δ* (MBY168), *cdc34-2* (KY1363), *ubc4Δ* (OKA196), *ubc5Δ* (OKA197), *ubc6Δ* (KY1606), *ubc7Δ* (OKA201), *ubc8Δ* (OKA198), *ubc10Δ* (OKA125), *ubc11Δ* (OKA199), and *ubc13Δ* (OKA200) strains were grown to saturation and used in a 10-fold dilution analysis starting with $3x10^8$ cell/ml on YPD or YPD containing 0.8 µg/ml CHX. Plates were incubated 2 or 6 days at 30°C.

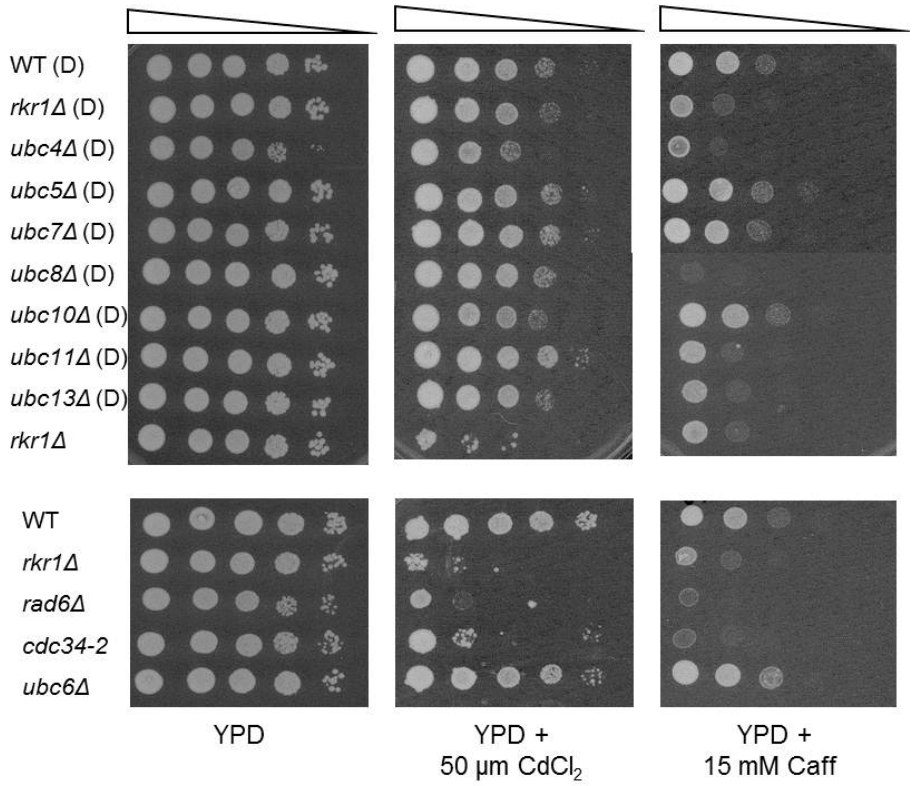

Figure 27: Some E2 mutants are unable to grow on media containing Cadmium Chloride or Caffeine.

The top panel of this figure represents strains from the deletion collection denoted as (D). All other strains are from the KY background. Wild type (D) (OY8), *ubc4Δ* (D) (OKA124), *ubc5Δ* (D) (OKA197), *ubc7Δ* (D) (OKA201), *ubc8Δ* (D) (OKA198), *ubc10Δ* (D) (OKA125), *ubc11Δ* (D) (OKA199), *ubc13Δ* (D) (OKA200), *rkr1Δ* (D) (in collection), wild type (KY665) *rkr1Δ* (SHY17), *rad6Δ* (BT132), *cdc34-2* (KY1363), and *ubc6Δ* (KY1607) strains were grown to saturation and used in a 10-fold dilution analysis starting with $1x10^8$ cell/ml on YPD or YPD containing 50 µM $CdCl_2$ or 15 mM Caffeine. Plates were incubated 2 or 3 days at 30^oC.

4.3.3 *In vivo* cross-linking and immunoprecipitation of Rkr1 to identify potential substrates and interactions

My genetic studies did not identify any one single E2 functionally interacting with Rkr1 in yeast, suggesting that Rkr1 could interact with multiple E2s within the cell. Due to its role in quality control, Rkr1 could also be involved in the degradation of specific proteins or a class of proteins, and could interact with multiple ubiquitin or proteasome components to accomplish this function. Any or all of these interactions may also be transient. Therefore, to identify possible substrates, partners, or interactors, and to give further insight into the function of Rkr1, I tried multiple methods of immunoprecipitation for Rkr1 to identify these interactions. This included a traditional TAP purification, as well as cross-linking and immunoprecipitation of HA-Rkr1 (data not shown). However, none of these methods revealed any detectable interactions for Rkr1 by silver-staining.

Dr. Richard Gardner (University of Washington) developed a method for identifying protein-protein interactions using cross-linking of cells with formaldehyde followed by quantitative mass-spectrometry of the total immunoprecipitated sample, rather than identification of proteins by visible bands on a silver-stained gel. This method was successfully used to confirm a substrate for the quality control ubiquitin ligase San1 (ROSENBAUM *et al.* 2011). Partnering with Dr. Gardner, I performed similar cross-linking using formaldehyde and a strain expressing either a FLAG-Protein-A-Rkr1 (modTAP-Rkr1) or untagged Rkr1 construct and followed with an immunoprecipitation of Rkr1 using IgG agarose. I first verified that Rkr1 could be detectably cross-linked to some cellular proteins following treatment with formaldehyde for varying amounts of time. This was indicated by a supershift in the size of Rkr1 using western analysis (Figure 28A). Next, I performed multiple cross-linking and immunoprecpitations of

modTAP- or untagged-Rkr1. Of these samples, a portion was checked for western analysis to verify the immunoprecipitation (Figure 28B). The remaining sample was run 1 cm into a 7.5% SDS POLYACRYLAMIDE gel in duplicate technical replicates of tagged or untagged lysate. I excised the gel slices containing protein and sent to Dr. Gardner them for further analysis by quantitative mass-spectrometry. This experiment was performed to this step in duplicate. A table of the top hits from one of these experiments is shown in Table 5.

As expected, of the proteins detected by mass-spectrometry, Rkr1 was the most abundant when comparing tagged with untagged samples. Dr. Gardner cross-referenced these results with results obtained from his experiments with San1 and Ubp10 (examples indicated in gray in Table 5) to identify proteins that had specific interactions with Rkr1 (indicated in white) with over 2-fold ratio of peptides identified from tagged vs. untagged Rkr1 samples. However, there was no consistency in top hits between the two duplicate experiments. Interestingly, many of the proteins obtained were ribosomal proteins. Rkr1 has been shown to purify with ribosomes in yeast, which would explain our results (BENGTSON and JOAZEIRO 2010). Although these experiments did not indicate any specific targets or interacting partners for Rkr1, the non-reproducible results do reinforce an important piece of information about Rkr1, in that Rkr1 is involved in quality control and therefore may not be expected to associate specifically and reproducibly with proteins.

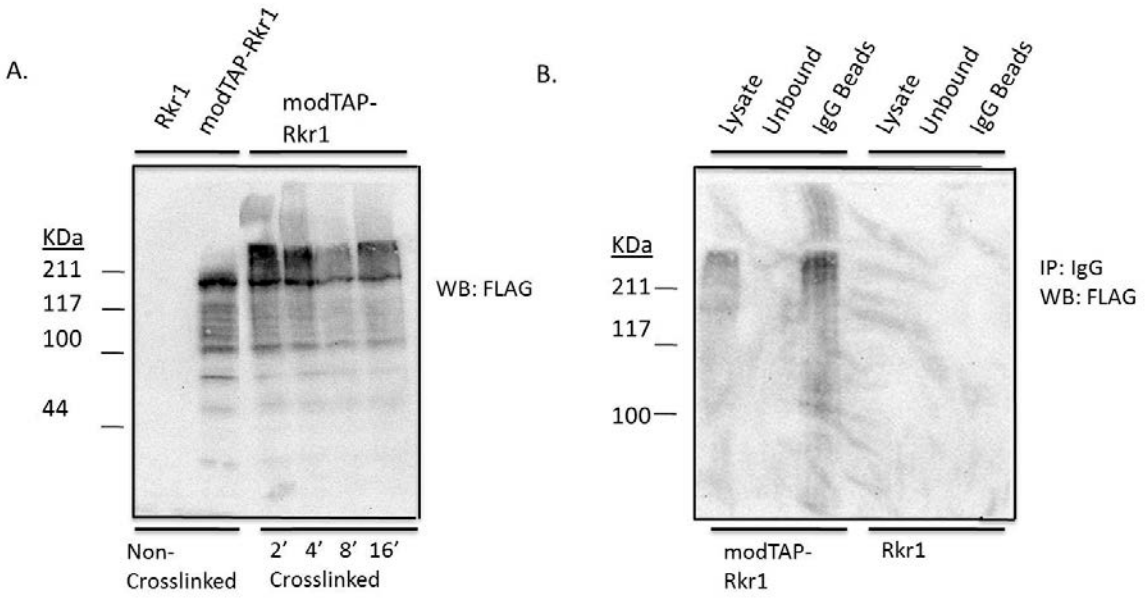

Figure 28: Cross-linking and immunoprecipitation of modTAP-Rkr1.

(A) *rkr1Δ* strains (MBY36) transformed with *RKR1/TRP1* (pPC65) or *modTAP-RKR1/TRP1* (pMB67) plasmids were grown to early log phase and fixed with 1% formaldehyde for the indicated amount of time before quenching. Extracts were made in SUME buffer and run on an 8% SDS POLYACRYLAMIDE gel before transfer to nitrocellulose. Western analysis to detect super-shifted and cross-linked Rkr1 was done with anti-FLAG antibody. (B) Extracts prepared as in (A) but cross-linked for 8 minutes before quenching were subjected to immunoprecipitation using IgG beads. A portion of this sample was analyzed by western analysis with anti-FLAG as in (A) to confirm immunoprecipitation, while the remainder of the sample was run on an 8% SDS POLYACRYLAMIDE gel 1 cm into the stacking and excised for quantitative mass-spectrometry.

4.3.4 Rkr1 is required for Htz1 ubiquitylation, but only when *HTZ1* is FLAG-tagged and overexpressed

In this chapter, I have so far described several approaches I have used to find partners and substrates for Rkr1. In addition to these methods, I have also used a targeted approach to investigate potential Rkr1 substrates as indicated by genetic interactions. The substrate that I investigated to the greatest degree was the histone H2A variant, Htz1.

In collaboration with Dr. Nevan Krogan at UCSF, we performed an Epistatic Miniarray Analysis (E-MAP) of a *rkr1Δ* strain (COLLINS *et al.* 2007). E-MAP analyzes the genetic interaction profiles of many genes to identify functional groups. This provided a genetic "profile" for *rkr1Δ*, which could then be compared to other known genetic profiles. Often, similar genetic profiles indicate genes that work in similar pathways, such as an enzyme and its substrate. Interestingly, *rkr1Δ* had a similar genetic profile to loss of *HTZ1*, which encodes a histone variant of H2A, as well as members of the Swr1 complex which catalyze the ATP-dependent exchange of Htz1 for canonical H2A (MIZUGUCHI *et al.* 2004). These results prompted experiments by Elia Crisucci and Mary Braun in our lab, who found that Rkr1 was required for the full ubiquitylation of FLAG-Htz1 when FLAG-Htz1 was overexpressed from a plasmid, suggesting that it may be a substrate for Rkr1 ubiquitin ligase activity. To test the specificity of Rkr1 for this ubiquitylation event, I asked if FLAG-Htz1 ubiquitylation required Bre1, another RING finger ubiquitin ligase with connections to chromatin (HWANG *et al.* 2003). Bre1 mediates H2B K123 ubiquitylation with the ubiquitin conjugase Rad6, a process which is dependent on Rtf1 of the Paf1 complex (WARNER *et al.* 2007; WOOD *et al.* 2003a; WOOD *et al.* 2003b).

FLAG-Htz1 was overexpressed in wild type, *rkr1Δ* and *bre1Δ* strains with a copper-inducible HIS-tagged or untagged ubiquitin plasmid. Proteins that were modified by HIS-Ub were immunoprecipitated from lysates made in denaturing conditions and the immunoprecipitated material was analyzed by western blotting for the presence of FLAG-Htz1. As expected from previous studies in the lab (Mary Braun and Elia Crisucci, unpublished data), *rkr1Δ* strains had decreased levels of FLAG-Htz1 in the ubiquitylated fraction of the cell lysate compared to wild type cells. However, loss of *BRE1* did not affect these levels of FLAG-Htz1 ubiquitylation (Figure 29). Although multiple ubiquitin ligases could be involved in targeting Htz1 for ubiquitylation when overexpressed, this result indicates that there is specificity to this assay and that not all RING finger ubiquitin ligases functionally or genetically linked to transcription are required for the quality control of Htz1.

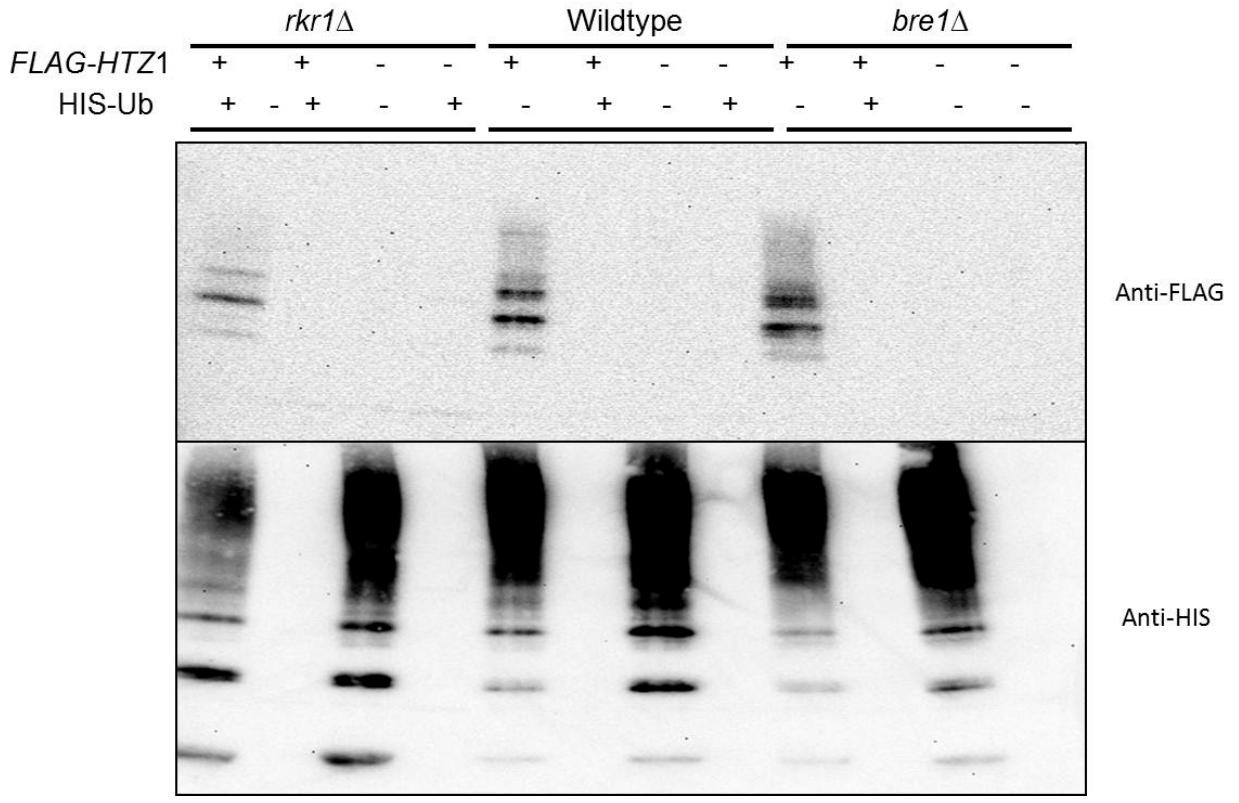

Figure 29: Rkr1 is specifically required for wild type levels of FLAG-Htz1 ubiquitylation when *FLAG-HTZ1* is overexpressed.

Wild type (KY306), *rkr1Δ* (MBY168), or *bre1Δ* (KY969) strains were transformed with a 2µ plasmid expressing FLAG-Htz1 (pMB82, *LEU2*) or empty vector (pRS425, *LEU2*) and either a 2µ plasmid containing HIS-Ubiquitin (pUB221, URA3/TRP1) or untagged ubiquitin (pUB175, URA3/TRP1) on a copper-inducible promoter. Strains were grown to early log phase in SC-Leu-Trp and were induced for 4 hours with 500 µM $CuSO_4$. Extracts were made using a denaturing buffer. Immunoprecipitation of all HIS-tagged ubiquitylated proteins was done using Ni-NTA agarose and these samples were run on a 7-20% gradient gel for western analysis with anti-FLAG M2 to detect Htz1 or anti-HIS to assess the efficiency of the immunoprecipitation.

4.3.5 Endogenous FLAG-Htz1 is not detectably ubiquitylated

We have shown that Rkr1 is required for the ubiquitylation of overexpressed FLAG-Htz1. Unlike monoubiquitylation, which is often associated with signaling, polyubiquitylation of Htz1 most likely indicates targeting for degradation and quality control (reviewed in (WEISSMAN 2001). Interestingly, the overexpression of *HTZ1* has been documented in breast cancer cells (HUA *et al.* 2008). However, the loss of another Paf1 complex member, *PAF1*, does not increase levels of *HTZ1* by microarray analysis (Elia Crisucci, unpublished results). Therefore, *rtf1Δ rkr1Δ* synthetic lethality is not easily explained by an aberrant increase in this histone variant.

It remained possible that an excess of Htz1 in the absence of *RTF1* could have lethal transcriptional consequences, so I next wanted to investigate if Rkr1 was important to the ubiquitylation of Htz1 when expressed at endogenous levels. To answer this question, *HTZ1-FLAG* was expressed from the endogenous locus in wild type and *rkr1Δ* cells. Strains were grown in the presence of the proteasome inhibitor MG132 to prevent protein degradation, and lysates were made in buffers containing a deubiquitinase inhibitor and protease inhibitors (LIU *et al.* 2007). A wild type strain expressing *FLAG-HTB1* (FLAG-H2B) was used as a positive control for ubiquitylation. Though we know H2B is monoubiquitylated at K123 as a signal for downstream histone methylation events, polyubiquitylation of this protein can be detected under some conditions (GENG and TANSEY 2008). I then performed an immunoprecipitation using FLAG-M2 agarose and western analysis for the ubiquitin on Htz1-FLAG or FLAG-H2B. As expected, my control FLAG-H2B was polyubiquitylated, and this could be detected both in the presence and absence of MG132 (Figure 30B; lanes *FLAG-H2B*, D and M). However, I was unable to detect any ubiquitylation signal of Htz1 in wild type or *rkr1Δ* lysates when expressed from the endogenous locus (Figure 30B; lanes *HTZ1-FLAG*, M).

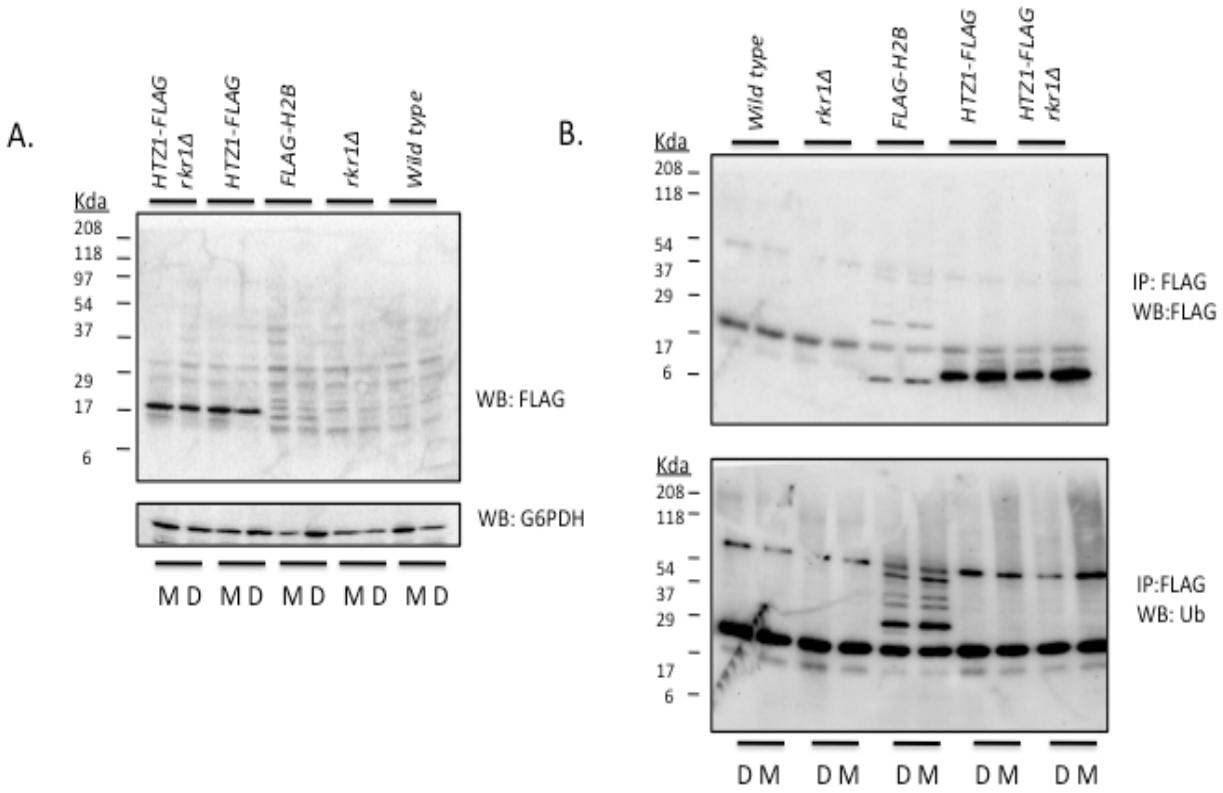

Figure 30: Endogenously expressed FLAG-Htz1 is not detectably ubiquitylated.

(A) Wild type or *rkr1Δ* strains expressing FLAG (KY1483, KY1488) or untagged Htz1 (KY306, MBY37) or FLAG-H2B (KY1044) were grown to early log phase in SC + Proline + 0/003% SDS to allow treatment with MG132 (M) or DMSO (D) for 30 minutes. Lysates were made with Buffer Ub (See Material and Methods) and analyzed by western analysis with anti-FLAG to check histone expression or anti-G6PDH as a loading control. (B) Lysates made as in (A) were subject to immunoprecipitation with anti-FLAG M2 agarose before detection by western analysis with anti-ubiquitin or anti-FLAG M2.

4.3.6 Overexpressed HA-Htz1 is not ubiquitylated in a Rkr1-dependent manner

The result that endogenous Htz1 is not detectably ubiquitylated led me to question whether Htz1 was a real target of Rkr1 under normal conditions, or an artifact of the overexpression system. When overexpressed, FLAG-Htz1 has been shown to immunoprecipitate with Rkr1, yet I was unable to detect a specific physical interaction between endogenously expressed Htz1 and Rkr1 in our strains ((MIZUGUCHI *et al.* 2004) and data not shown). Therefore, Rkr1 may only target Htz1 when Htz1 is at high levels. Conversely, because Rkr1 is involved in general quality control of proteins, my results so far may have indicated that Htz1 is not a specific target of Rkr1.

To date, nonstop proteins are the only targets of Rkr1 that have been established (BENGTSON and JOAZEIRO 2010; WILSON *et al.* 2007). Notably, Rkr1 most likely recognizes the poly-lysine tail of a nonstop protein which is a result of translational read-through into the poly(A) tail (BENGTSON and JOAZEIRO 2010). Histones are naturally lysine rich, as these residues are often targets of modifications such as methylation and ubiquitylation. In addition to the fact that Htz1 contains multiple lysines, there was a concern that the FLAG tag of Htz1, which contains 6 lysines per 3XFLAG sequence, could mimic a poly-lysine tail of a nonstop protein. FLAG tags can also be non-specific targets of ubiquitylation (Dr. Richard Gardner, personal communication). Therefore, to assess if Rkr1 was targeting Htz1 specifically, or through the FLAG tag, Dr. Margaret Shirra created an HA-tagged 2µ construct for overexpressing Htz1. Using this plasmid, I used the HIS-ubiquitin immunoprecipitation assay as above to investigate the levels of ubiquitylation of HA-Htz1 in wild type and *rkr1Δ* cells. Interestingly, I found that while HA-Htz1 is targeted for polyubiquitylation, this event is not dependent on the presence of Rkr1 in yeast (Figure 31).

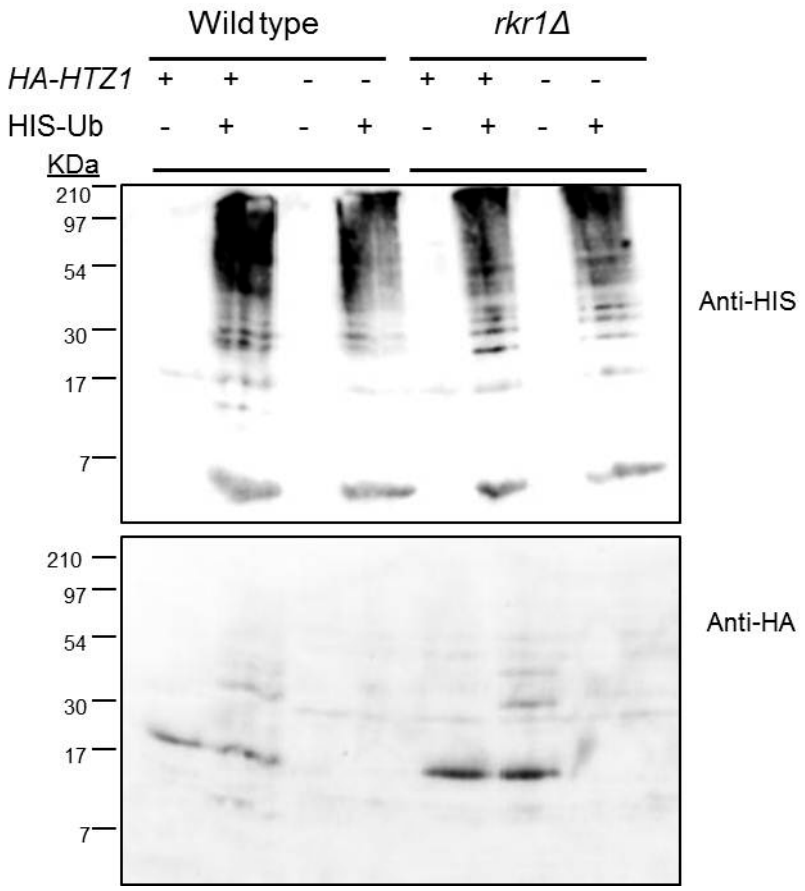

Figure 31: *RKR1* is not required for HA-Htz1 ubiquitylation when *HA-HTZ1* is overexpressed.

Wild type (KY665) and *rkr1Δ* (SHY11) strains were transformed with *HA-HTZ1* (*LEU2*) plasmid or empty vector (pRS425, *LEU2*) and HIS-tagged (pUB221-*URA3*, *TRP1*) or untagged ubiquitin (pUB175-*URA3*, *TRP1*) plasmids with a copper-inducible promoter. Strains were grown to log phase in SC-Leu-Trp and induced with 500 μM $CuSO_4$ for four hours. Lysates were made under denaturing conditions. Immunoprecipitations were performed against all ubiquitylated proteins using Ni-NTA agarose and western analysis was performed with anti-HIS to assess efficiency of ubiquitin immunoprecipitation or anti-HA to assess levels of ubiquitylated Htz1 in immunoprecipitation.

4.4 DISCUSSION

The holy grail of investigating an E3 is identifying its interactions and targets and understanding its cellular role. To this end, I discussed in this chapter multiple methods I have used to detect substrates and partners for Rkr1-mediated ubiquitylation which have included both directed and global methods of analysis. Though at the time of these experiments, we were not aware of the role of Rkr1 in nonstop protein quality control, it is possible and likely that other Rkr1 interactions still remain to be found.

Using phenotypic analysis, I was able to assign new phenotypes to *rkr1* mutant strains and use these data to explore potential interactions with Rkr1. In a genetic search for the ubiquitin conjugase partner or E2 for Rkr1, I found similar cycloheximide, inositol, cadmium chloride, and caffeine sensitivities for strains mutated in *RKR1*, *CDC34*, *RAD6*, and *UBC4*. Therefore, these genes may encode the most likely candidates for a Rkr1 E2 partner. In agreement with this conclusion, all of these E2s are found in the cytoplasm where Rkr1 is also predominantly localized (BENGTSON and JOAZEIRO 2010). Because Rad6 is required for ubiquitylation of H2B at K123, which is an Rtf1-dependent event (HWANG *et al.* 2003; NG *et al.* 2003a; WOOD *et al.* 2003b), the phenotypic similarities between *rad6Δ* and *rkr1Δ* are most likely due to its role in a pathway parallel to Rkr1 (BRAUN *et al.* 2007), especially considering that loss of *RTF1* confers the same phenotypes. While a mutation in *CDC34* confers phenotypes more similar to loss of *RKR1*, Ubc4/Ubc5 have been identified as physical interactors with the human RING domain of Rkr1, and therefore are also very plausible candidates (VAN WIJK *et al.* 2009). I may not be able to observe *rkr1Δ*-related phenotypes in a *ubc4Δ* or *ubc5Δ* strain due to the partial redundancy of Ubc4 and Ubc5 in yeast (SEUFERT and JENTSCH 1990; STOLL *et al.* 2011). However, a strain containing mutations in both of these genes is inviable or extremely sick, and

therefore cannot be used for these studies. In addition, because I was unable to include it in my analysis, I cannot discount that the essential E2 Ubc1 is also involved in a Rkr1 interaction. The absence of a nonstop phenotype for any of the E2 mutants tested suggests that either none of these E2s interact with Rkr1, or that Rkr1 has multiple partners with which it functions.

In addition to phenotypic analyses, I have also tried other unbiased methods to identify novel Rkr1 interactions and potential substrates. These methods include purification of Rkr1 and potential interacting proteins under cross-linking conditions. Although these methods were successful in identifying Rkr1 and a range of interactions, which included ribosomal proteins, this method did not reveal any specific or reproducible interactions that may have suggested specific substrates for Rkr1-dependent degradation. In fact, the results would indicate Rkr1 is involved broadly in quality control and does not specifically target a subset of proteins, but rather a class of proteins which may occur under conditions of stress such as in the absence of *RTF1*. The recent findings that Rkr1 is involved in nonstop protein quality control, as well as my results in earlier chapters, further support this hypothesis (BENGTSON and JOAZEIRO 2010). For future experiments involving this cross-linking method, I have created two additional modTAP constructs of Rkr1, which include a deletion of the conserved N-terminal region or RING domain. I have not yet used these constructs in this experiment, but they may provide additional clues into Rkr1 function by capturing interactions without allowing ubiquitylation and/or degradation of a substrate. In addition, performing these experiments in the absence of *RTF1* may provide the stress needed to enhance Rkr1-specific interactions. However, it is very likely that as a quality control ubiquitin ligase, if in fact Rrk1 targets a group of substrates rather than individual and normal proteins, then this method may only identify transient interactions necessary to degrade these proteins such as E2, proteasome, or ribosomal subunits and partners.

Additional methods of investigating a pathway for Rkr1 function included a collaboration with Dr. Nevan Krogan to obtain an epistatic miniarray profile (E-MAP) for *rkr1Δ* strains. Often, substrates and enzymes in these studies will exhibit a similar genetic profile. We found that *rkr1Δ* strains have a similar genetic profile to strains deleted of *HTZ1* or members of the *SWR1* complex, which led us to hypothesize that Htz1 is a possible substrate for Rkr1. Htz1 as a potential target for Rkr1 ubiquitylation was first analyzed by Mary Braun and Elia Crisucci. However, I showed in this chapter that, although another RING finger ubiquitin ligase, Bre1, is not required for FLAG-Htz1 ubiquitylation when *HTZ1* is overexpressed, Rkr1 is only required for targeting this protein when this protein is FLAG-tagged. This may be an artifact, resulting from Rkr1 recognizing the lysines in the FLAG tag as being similar to a poly-lysine tail of a nonstop protein (BENGTSON and JOAZEIRO 2010). Additionally, I showed that endogenous Htz1 is not detectably ubiquitylated under conditions of proteasome inhibition.

Our E-MAP analysis functionally clustered Rkr1 with Htz1 and the Swr1 complex. Interestingly, we have since learned that Htz1 also plays a modest role in regulating nonstop protein levels (Chapter 3 and (WILSON *et al.* 2007)). In the E-MAP studies, the second complex that displayed a genetic profile similar to that of Rkr1 was the elongator (ELP) complex, which has been implicated both in transcription elongation and in tRNA modification (reviewed in (SVEJSTRUP 2007). Although this connection could also indicate that the ELP proteins are targets for Rkr1 ubiquitylation, the elongator complex is also required for proper translational fidelity and recognition of stop codons through its role in tRNA modification (HUANG *et al.* 2005). Therefore, the E-MAP results may have been indicating the role of Rkr1 in protein quality control, rather than potential substrates.

Finding partners and substrates for a ubiquitin ligase, whose role is to degrade proteins, is an arduous task. To find physical interactions for Rkr1, I have used both directed and unbiased methods and a variety of innovative techniques and genetic approaches. However, as an E3 involved in the degradation of aberrant proteins, Rkr1 most likely interacts transiently with a wide array of proteins in the cell and these interactions can be difficult to detect. Although the data presented here did not identify new interactions for this ubiquitin ligase, the results all show in an overwhelming support for Rkr1 in quality control, where new interactions most likely depend on the ever-changing stress conditions of the cell.

Table 4: Summary of E2 phenotypes and genetics

(Strain) KY indicates strains from the Arndt lab collection. D indicates strains from the deletion collection. (Localization) N = Nucleus, C = Cytoplasm, ER = Endoplasmic Reticulum, P = Peroxisome. N/D = Not done. N/A = Not applicable. (+) indicates degree of growth while (-) indicates no growth at all. The light gray shading denotes reference strains for phenotypes, while the pink shading indicates E2 mutations. Studies with the essential gene *UBC1* were not performed.

Gene		Localization	0.8 μg/ ml CHX	SD-INO	*his3* nonstop phenotype	15 mM Caffiene	50 μM $CdCl_2$	*Crossed by rtf1*Δ [*PSI*$^+$]	*Crossed by rkr1*Δ [*PSI*$^+$]
WT	KY	N/A	++++	++++	-	++++	++++		
	D	N/A	++++	N/D	-	++++	++++		
*rkr1*Δ	*KY*	N	+	-	+++	++	+ [*PSI*$^+$] only	inviable	
	D	N	+	N/D	+++	++	++++		
*rtf1*Δ	*KY*	N	+	+++	-	++	+		inviable
*ubc1*Δ = *lethal*		N/C							
cdc34-2	*KY*	N/C	++	+	-	++	++	?	sick
*rad6*Δ	*KY*	N/C	-	+	-	++	++	normal	sick
*ubc4*Δ	*D*	N/C	++	+++	-	++	+++	normal	normal
*ubc5*Δ	*D*	N/C	+++	++++	-	++++	++++	normal	normal
*ubc6*Δ	*KY*	ER	+++++	++++	-	++++	++++	N/D	N/D
*ubc7*Δ	*D*	ER	++++	++++	-	++++	++++	N/D	N/D
*ubc8*Δ	*D*	C (N)	++++	++++	-	+	++++	N/D	N/D
*ubc10*Δ	*D*	P	++++	++++	-	++++	++++	N/D	N/D
*ubc11*Δ	*D*	?	++++	++++	-	++	++++	N/D	N/D
*ubc13*Δ	*D*	C (N)	++++	++++	-	++	++++	N/D	N/D

Table 5: Mass-Spec results from Rkr1 Cross-linking and IP

Number of peptides displayed (Tagged or Untagged) is a summary of peptides identified from three individual immunoprecipitations within one experiment for either the tagged or untagged Rkr1 construct. The results were ranked by Tagged vs Untagged peptides and the top 20 identified proteins are shown here. Gray shading indicates proteins also purified with San1 and Ubp10 (considered background). Rkr1 is highlighted in teal.

Protein	TAG-GED	UNTAG-GED	TAG/UNTAG
RKR1	52	0	#DIV/0!
RPL5	8	0	#DIV/0!
RPS23A	6	0	#DIV/0!
TDH2	10	1	10
IDS2	8	1	8
TKL1	11	2	5.5
FAS1	20	4	5
GCD11	5	1	5
URB1	5	1	5
VMA1	5	1	5
ARF1	8	2	4
RPL10	8	2	4
RPS2	8	2	4
RPP0	22	6	3.6
RPS22A	7	2	3.5
TEF4	25	8	3.1
ENO1	12	4	3
GPB1	12	4	3
RPL14B	6	2	3

Table 6: *S. cerevisiae* strains used in Chapter 4

Strain	Genotype
SHY11	MAT α *rkr1Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 trp1Δ63 his4-9128δ ura3-52*
SHY17	MAT a *rkr1Δ::HIS3 his3Δ200 leu2Δ1 trp1Δ63*
BTY132	MAT a *rad6Δ::KanMX his3Δ200 leu2Δ1 ura3Δ0*
ECY1	MAT a *rtf1Δ::KanMX*
MBY6B	MAT a *rkr1-SL505 leu2Δ1 trp1Δ63 ade2 ade3*
MBY29	MAT a *rkr1Δ::KanMX his3Δ200 leu2Δ1 trp1Δ63*
MBY36	MAT α *rkr1Δ::KanMX his3Δ200 trp1Δ63*
MBY37	MAT α *rkr1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
MBY168	MAT a *rkr1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY306	MAT α *lys2-173R2 ura3-52 leu2Δ1 trp1Δ63*
KY665	MAT α *rkr1Δ::HIS3 his3Δ200 lys2-128δ leu2Δ1 trp1Δ63 his4-9128δ ura3-52*
KY931	MAT a *rad6Δ::URA3 his4-912δ leu2Δ1 ura3-52*
KY969	MAT α *bre1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY1044	MAT a *hta1-htb1Δ::LEU2 hta2-htb2Δ::TRP1 his3Δ200 leu2Δ1 ura3-52 trp1Δ63 [pJH23= HIS3/HTA1-FLAG-HTB1/C/A]*
KY961	MAT α *bre1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY1483	MAT α *HTZ1-FAG::KanMX lys2-173δ leu2Δ1 ura3-52*
KY1488	MAT a *HTZ1-FLAG::KanMX rkr1Δ::KanMX lys2-173δ leu2Δ1 ura3-52 trp1Δ63*
KY1198	MAT α *his3Δ200 leu2Δ1 trp1Δ63*
KY1363	MAT α *cdc34-2 his3Δ200 leu2Δ1 ura3-52*
KY1606	MAT α *ubc6Δ::KanMX his3Δ200 leu2Δ1 ura3-52 lys2-128δ trp1Δ63 ade8Δ*
KY1607	MAT a *his3Δ200 leu2Δ1 ura3-52 lys2-128δ*
OY8	MAT a *his3Δ1 leu2Δ0 ura3Δ met15Δ0*
OKA125	MAT a *ubc10Δ::KanMX his3Δ1 leu2Δ0 ura3Δ met15Δ0*
OKA196	MAT a *ubc4Δ::KanMX his3Δ1 leu2Δ0 ura3Δ met15Δ0*
OKA197	MAT a *ubc5Δ::KanMX his3Δ1 leu2Δ0 ura3Δ met15Δ0*
OKA198	MAT a *ubc8Δ::KanMX his3Δ1 leu2Δ0 ura3Δ met15Δ0*
OKA199	MAT a *ubc11Δ::KanMX his3Δ1 leu2Δ0 ura3Δ met15Δ0*
OKA200	MAT a *ubc13Δ::KanMX his3Δ1 leu2Δ0 ura3Δ met15Δ0*
OKA201	MAT a *ubc7Δ::KanMX his3Δ1 leu2Δ0 ura3Δ met15Δ0*

5.0 CONCLUSIONS AND FUTURE DIRECTIONS

Rkr1 is a ubiquitin ligase required for the ubiquitylation and degradation of nonstop proteins, which arise from translational read-through of normal stop codons or from translation of nonstop mRNAs that lack stop codons (BENGTSON and JOAZEIRO 2010; WILSON *et al.* 2007). Both these mRNAs and the resulting proteins are normally targets of quality control. Rkr1 was first identified and characterized following a genetic screen for genes that were essential in the absence of the transcription elongation factor, Rtf1 (BRAUN *et al.* 2007). As a member of the Paf1 complex, Rtf1 accompanies RNA Pol II across actively transcribed open reading frames (MAYER *et al.* 2010). Rtf1 is also required for H2B ubiquitylation on K123, a conserved modification whose correct patterning is important to both the activation and repression of several genes (NG *et al.* 2003a; TOMSON *et al.* 2011; WARNER *et al.* 2007; WOOD *et al.* 2003b). Interestingly, Rkr1 is required in the absence of this specific Rtf1 function, connecting the pathways of histone modification to protein quality control (BRAUN *et al.* 2007).

5.1 MUTATIONS THAT SUPPRESS SYNTHETIC LETHALITY BETWEEN DELETIONS OF RTF1 AND RKR1

To better understand the requirement for Rkr1 in the absence of Rtf1-mediated H2B ubiquitylation, I performed a screen to identify suppressors of *rtf1Δ rkr1Δ* synthetic lethality

using a *LEU2*-marked transposon mutagenesis library (KUMAR *et al.* 2000). Suppressors of *rtf1Δ rkr1Δ* synthetic lethality could function in several ways. For example, synthetic lethality in *rtf1Δ rkr1Δ* strains could be due to a buildup in one Rkr1 substrate. In this case, mutation of the gene encoding this substrate could rescue lethality in these strains by decreasing substrate levels. Alternatively, mutations identified by this screen could result in suppression of a *rtf1Δ* or *rkr1Δ*-specific defect in these cells. I screened over 15,000 transformants, originally identifying over 50 candidates that were further analyzed for their ability to rescue lethality as a result of a single transposon insertion. This resulted in 15 candidates that were used in a backcross to verify that no additional mutation was present that could suppress *rtf1Δ rkr1Δ* synthetic lethality independent of the *LEU2*-marked transposon. Three transformants all passed this criteria, and the transposon insertions were mapped using plasmid rescue and sequencing (BURNS *et al.* 1994). In this thesis, I further characterize and describe these mutations in *HSP104*, *CHL1*, and *RPC17*.

5.1.1 *HSP104*

A transposon insertion in the 3' coding region of *HSP104* was able to rescue *rtf1Δ rkr1Δ* lethality. Hsp104 is required for the proper disaggregation of misfolded proteins following heat shock, as well as the propagation of yeast prions (reviewed in (GRIMMINGER-MARQUARDT and LASHUEL 2010)). Prions are insoluble and infectious protein aggregates that are inherited by daughter cells but can also appear *de novo*. Hsp104 breaks prion aggregates into smaller infectious particles which seed and recruit soluble protein in order to propagate the prion (reviewed in (GRIMMINGER-MARQUARDT and LASHUEL 2010)). It has been shown that mutations that disrupt the ATPase domain of Hsp104, which lies in the C-terminal region of the protein, impair the ability of Hsp104 to propagate prions and results in their clearance from yeast cells

(CHERNOFF *et al.* 1995; MACKAY *et al.* 2008). Accordingly, I have shown that the transposon insertion I identified from my genetic screen is able to clear cells of the prion [*PSI*$^{+}$], an aggregate of the essential translation termination factor Sup35.

Yeast have several identified and potential prions (reviewed in (CROW and LI 2011)). Of these prions, I confirmed that it was the presence of [*PSI*$^{+}$] specifically that led to synthetic lethality between *rtf1Δ* and *rkr1Δ* using several mechanisms. Although Hsp104 is required for general yeast prion propagation, it has been shown that the overexpression of *HSP104* can uniquely clear cells of [*PSI*$^{+}$] (CHERNOFF *et al.* 1995). In accordance with this, I found that both the deletion and overexpression of *HSP104* rescued *rtf1Δ rkr1Δ* synthetic lethality. In addition, I showed that inactivating Hsp104 by growing yeast in the presence of guanidine hydrochloride (FERREIRA *et al.* 2001; JUNG and MASISON 2001) also rescued synthetic lethality. I also verified results from a previous genetic screen by Mary Braun to identify genes that suppressed *rtf1Δ rkr1Δ* lethality when overexpressed. In this screen, Mary Braun isolated several plasmids that contained multiple open reading frames. Interestingly, three genes contained on these plasmids were *HSP104*, *URE2* and *LSM4*, which encode the yeast prions [*URE3*] and [*LSM4*], respectively. Prions can have both positive and negative interactions (reviewed in (DERKATCH and LIEBMAN 2007)), and I showed that overexpression of these prion-encoding genes caused clearance of [*PSI*$^{+}$] and rescue of *rtf1Δ rkr1Δ* lethality. Lastly, I proved that this prion caused lethality between *rtf1Δ* and *rkr1Δ* by transferring [*PSI*$^{+}$] -containing cytoplasm to viable [*psi*$^{-}$] *rtf1Δ rkr1Δ* cells, which resulted in cell death.

As a Sup35 aggregate, [*PSI*$^{+}$] causes multiple phenotypes in yeast. [*PSI*$^{+}$] cells are unable to efficiently recognize stop codons, resulting in translational-read through of normal stop codons as well as nonsense stop codons (WILSON *et al.* 2005). This results in proteins that are

now targets of quality control pathways. In addition, efficient translation termination is necessary to recruit degradation machinery specific for aberrant mRNAs, such as nonsense and nonstop mRNAs. Therefore, without sufficient soluble Sup35, there is an increase in these defective transcripts, presenting an additional problem in quality control for the cell (TRUE *et al.* 2004; WILSON *et al.* 2005). One of the consequences of [*PSI*$^+$], nonstop proteins, are targets of Rkr1-mediated ubiquitylation and degradation (BENGTSON and JOAZEIRO 2010). I showed that the presence of [*PSI*$^+$] negatively influences *rkr1Δ* phenotypes and genetic interactions. In addition, using a *sup35-Y351C* mutation that mimics the nonsense suppression phenotype of [*PSI*$^+$], I showed that it was the inability to recognize stop codons that led to the *rkr1Δ* phenotype of cadmium chloride sensitivity.

Interestingly, however, I also observed that although the *sup35-Y351C* allele caused slight sickness in [*psi*$^-$] *rtf1Δ rkr1Δ* cells, it did not cause synthetic lethality. There are several explanations for this result. First, the mutation may not completely recapitulate nonsense suppression to the levels of [*PSI*$^+$] in our strains. Second, there may be additional phenotypes of [*PSI*$^+$] not mimicked by this allele, such as mRNA quality control problems, that also contribute to synthetic lethality. In the future, as the requirement for *RTF1* in [*PSI*$^+$] *rkr1Δ* cells is better understood, this explanation may become clearer. For example, the absence of *RTF1* may lead to additional mRNA quality control problems such as an increase in improperly processed transcripts. Alternatively, the loss of *RTF1* may cause a decrease in transcript levels for proteins that are necessary to combat the excess problems in quality control in [*PSI*$^+$] *rkr1Δ* strains. Lastly, the presence of cellular protein aggregates such as [*PSI*$^+$] may present a physical quality control problem to these cells. However, because the presence of other prions, such as [*PIN*$^+$], does not induce *rtf1Δ rkr1Δ* synthetic lethality, this is a less likely scenario.

Given my results, it may also be beneficial to isolate and characterize the remaining 12 transposon insertion mutations I originally identified from my screen. I initially put these mutations aside, because I saw spontaneous growth of *rtf1Δ rkr1Δ* spores without linkage to the transposon insertion in the final genetic cross. However, in the process of confirming the role of the non-Mendelian genetic element [*PSI*$^{+}$] in *rtf1Δ rkr1Δ* synthetic lethality, I have observed several instances of *rtf1Δ rkr1Δ* viability, particularly during tetrad analysis. I have not correlated this viability with the presence or absence of [*PSI*$^{+}$], but I find this result varies with the strains used for the genetic cross. Levels of [*PSI*$^{+}$] within strains can vary in strength, and therefore it is very possible that loss of [*PSI*$^{+}$] in a spore during meiosis could result in viability when *rtf1Δ* and *rkr1Δ* co-segregate. In addition, I have shown that diploids heterozygous for *hsp104Δ* are able to rescue *rtf1Δ rkr1Δ* synthetic lethality, even without co-segregation of *hsp104Δ* with *rtf1Δ rkr1Δ*, suggesting that *hsp104Δ* is a dominant suppressor. For these reasons, the 12 transposon insertions that I did not further investigate could prove to have valid suppressors as well.

5.1.2 *CHL1*

Another genetic suppressor of *rtf1Δ rkr1Δ* synthetic lethality that I describe in this thesis is mutation in the sister chromatid cohesion gene *CHL1*. Chl1 in yeast is a putative DNA helicase required for proper sister chromatid cohesion during S phase and proper chromosome segregation during meiosis II (MAYER *et al.* 2004; PETRONCZKI *et al.* 2004; S 2000). Deletion of *CHL1* suppresses a *maml1Δ* defect in kinetochore attachment by compromising sister chromatid cohesion and allowing successful meiotic division (PETRONCZKI *et al.* 2004). Chl1 has also been implicated in telomeric silencing and rDNA recombination (DAS and SINHA 2005). Loss of *CHL1* causes DNA damage and replication phenotypes (LAHA *et al.* 2011; LAHA *et al.* 2006).

At this time, it is unclear why a *chl1* mutation rescues *rtf1Δ rkr1Δ* synthetic lethality. I have shown that this is not due to the clearance of [*PSI*$^{+}$], and therefore this suppression mechanism is different than that of an *hsp104* mutation. When I was further exploring the effect of *rtf1Δ* on nonstop protein synthesis in cells, I found that it decreased the levels of these proteins. Although the mechanism of this is not currently known, I did show that deletion of *CHL1* opposed this *rtf1Δ* defect. In addition, the loss of *RTF1* causes a decrease in the levels of expression from the nonsense allele *ade1-14* (STRAWN *et al.* 2009). I show in my studies that a *chl1* mutation opposes this defect as well, partially suppressing this phenotype and causing an increase in Ade1 protein. These results provide an avenue for further study of this newly identified connection between Rtf1 and Chl1.

Given that *chl1Δ,* a suppressor of *rtf1Δ rkr1Δ* synthetic lethality, opposes *rtf1Δ*-specific phenotypes, I hypothesize that it rescues lethality by suppressing an *rtf1Δ* defect rather than a defect in *rkr1Δ*. The loss of *RTF1* is most likely affecting levels of nonsense and nonstop reporter proteins at the level of transcription or processing, which could have negative consequences on export, stability, or translatability of the transcript. I have used the Protein A nonstop reporter to measure levels of nonstop mRNA in multiple strains using northern analysis, but this reporter is on a high-copy number plasmid and the levels of expression vary widely even in wild type strains. Therefore, it still remains to be identified whether *rtf1Δ* affects nonstop transcript levels. Interestingly, it has been shown that Ctr9 of Paf1c is required for proper poly(A) site usage of the *ade1-14* transcript (STRAWN *et al.* 2009). In the future, it would be interesting to validate this result in *rtf1Δ* strains, and to see if the absence of *CHL1* suppresses this result by correcting the poly(A) site. It is possible that by choosing an earlier or later poly(A) site for cleavage, the loss of *RTF1* could actually create new nonstop or nonsense targets that

cannot be appreciated by the reporters I've used thus far. These aberrant mRNAs could lead to quality control problems that would be detrimental in [*PSI*$^{+}$] *rkr1Δ* cells. The most effective way to analyze this problem would be a genome-wide or targeted analysis of poly(A) sites in *rtf1Δ* cells, possibly by enriching for nonsense and nonstop transcripts using a *upf1Δ* or *ski7Δ* background, respectively. Candidate genes affected in this manner by loss of *RTF1* could then be tested for suppression by loss of *CHL1*. Rkr1 is required in the absence of Rtf1, Xrn1, and Spt10 (This thesis) (BRAUN *et al.* 2007). Therefore, the types of transcripts that are improperly processed or mis-regulated by loss of *RTF1* may be further clarified or identified by comparing microarray data from wild type, *rtf1Δ*, *xrn1Δ*, and *spt10Δ* cells.

Chl1 is required for proper sister chromatid cohesion (SCC) (MAYER *et al.* 2004). Interestingly, Rtf1-mediated H2B ubiquitylation is required for methylation of the kinetochore protein Dam1 by the H3 K4 methyltransferase Set1 (LATHAM *et al.* 2011; ZHANG *et al.* 2005b), a process which could also affect sister chromatid cohesion. Dam1 methylation inhibits Dam1 phosphorylation by Aurora B/Ipl1 (ZHANG *et al.* 2005b). Lower levels of Dam1 phosphorylation cause defects in chromosome segregation (CHEESEMAN *et al.* 2002). Interestingly, loss of SCC causes an increase in Dam1 phosphorylation. Although the effects of this extended phosphorylation are not clear, it may be important to kinetochore detachment (KEATING *et al.* 2009). Therefore, it would also be interesting to analyze the role of Rtf1 in Chl1-related defects, such as SCC. The specific role for Chl1 in SCC is not currently known. However, one hypothesis might be that it is required to recognize an event downstream from H2B ubiquitylation necessary for SCC. In the absence of *RTF1*, H2B K123 ubiquitylation and Dam1 methylation does not occur (LATHAM *et al.* 2011; ZHANG *et al.* 2005b). The loss of *CHL1* might bypass this requirement for Dam1 methylation, leading to suppression of these *rtf1Δ*-specific

defects. Alternatively, a *chl1Δ* mutation may suppress the *rtf1Δ*-defect and restore Dam1 methylation. To test these hypothesizes, I would assess levels of Dam1 methylation in *rtf1Δ chl1Δ* strains compared to *rtf1Δ* single mutants, which lack Dam1 methylation. In addition, I would also test a possible role for Rtf1 in SCC. Lastly, to gather more information about the connection between these genes, I would test *rtf1Δ chl1Δ* strains for suppression of multiple *chl1Δ* or *rtf1Δ* phenotypes by dilution analysis. These phenotypes would include HU and MMS sensitivity (indications of DNA replication and damage defects), caffeine, cycloheximide, and caffeine (general stress), as well as benomyl (mitotic defects).

5.1.3 *RPC17*

The third genetic suppressor of *rtf1Δ rkr1Δ* synthetic lethality that I identified was a mutation within the 3' UTR of *RPC17*. Like the mutation in *CHL1*, an *rpc17* strain does not clear cells of [*PSI*$^{+}$]. *RPC17* is an essential gene that encodes a subunit of RNA Pol III (FERRI *et al.* 2000). I confirmed this genetic suppressor using an *rpc17-DAmP* allele that I obtained from Open Biosystems (BRESLOW *et al.* 2008). This allele also contains an insertion within the 3' UTR, which has been shown to lower transcript levels, likely by targeting them for degradation (BRESLOW *et al.* 2008). Therefore, it is reasonable to hypothesize that lower levels of *RPC17* transcript, and hence lower levels of Rpc17 protein, result from a 3' UTR mutation. However, this has yet to be confirmed. In addition, I hypothesize that the lower levels of this transcript cause a decrease in RNA Pol III function, possibly resulting in a decrease in tRNA levels and a decrease in translation efficiency.

I have tested the ability of the *rpc17* mutation to suppress multiple *rtf1Δ* or *rkr1Δ* phenotypes or genetics. Thus far, the only phenotype slightly suppressed by an *rpc17-DAmP*

allele is 6AU sensitivity in *rtf1Δ* cells (SQUAZZO *et al.* 2002). 6AU depletes the cellular pool of nucleotides, which has been argued to cause RNA Pol II stalling and transcription elongation defects (EXINGER and LACROUTE 1992). This suggests that loss of RNA Pol III activity might in some way affect RNA Pol II elongation. One possible explanation for this may be that decreased Pol III transcription means less use of the free nucleotide pool, and therefore more available for use by Pol II.

By affecting levels of tRNAs, lower levels of *RPC17* transcripts may indirectly affect translation. Translation may be slowed, or it may not efficiently recognize certain codons. It is unclear how this could rescue *rtf1Δ rkr1Δ* lethality. However, if *rtf1Δ rkr1Δ* synthetic lethality is caused by an increase in aberrant mRNA that creates an increase in aberrant protein that is unable to be degraded in the absence of *RKR1*, then decreased translation may ease this burden. To test this idea, I would first investigate if the *rpc17-DAmP* allele causes a decrease in tRNA levels. I would follow this with experiments to identify the effect of this allele on translation efficiency.

Lastly, several other connections to translation have been identified in our lab. In the genetic screen performed by Mary Braun that identified high-copy suppressors of *rtf1Δ rkr1Δ* lethality, which led to my experiments on *LSM4* and *URE2*, there were also multiple translation-related genes contained on these plasmids. These included *THS1*, which encodes a threonyl-tRNA synthetase, and two separate plasmids that contained tRNA(Ala) genes. These genes must be individually cloned into overexpression vectors to determine if they rescue *rtf1Δ rkr1Δ* synthetic lethality. In addition, in the E-MAP studies performed in collaboration with Dr. Nevan Krogan on *rkr1Δ* strains, it was determined that loss of *RKR1* has a similar genetic profile to strains lacking Elongator subunits (Elp1-6; unpublished data). Elongator has been associated

with transcription elongation in yeast and human cells, and is also necessary for maintaining levels of the chaperone Hsp70 in mammalian cells (SUAUD *et al.* 2011) (reviewed in (SVEJSTRUP 2007)). In addition, it has an established role in tRNA modification and translational fidelity (reviewed in (SVEJSTRUP 2007)). Given these results and the connections between Rkr1 and nonstop proteins, as well as prions and chaperones, it is possible that *rtf1Δ* or *rkr1Δ* strains, or both, are greatly influenced by changes in translation and chaperones.

5.2 TARGETS AND PHYSICAL INTERACTIONS OF RKR1

When Rkr1 was first characterized by our lab, it was identified by its genetic interaction with a transcription elongation factor (BRAUN *et al.* 2007). In subsequent years, it has been described by its role in nonstop protein quality control (BENGTSON and JOAZEIRO 2010; WILSON *et al.* 2007). During my thesis work, I sought to better understand this ubiquitin ligase and its requirement in the absence of *RTF1* by first identifying interacting partners or substrates. By nature of its role as a ubiquitin ligase, most of these physical connections are most likely transient. Therefore, I tried immunoprecipitation of Rkr1 both in uncross-linked and cross-linked conditions to capture these transient interactions. However, I was unable to consistently identify any proteins from these methods. In light of the discovery that Rkr1 is a quality control ubiquitin ligase, this result may not be surprising. Not only would substrates be transiently interacting with a quality control E3 prior to degradation, the substrates likely vary among growth conditions and are not expected to be enriched for a particular protein.

In addition, I also used phenotypic and genetic analyses to identify E2 partners for Rkr1, but this method revealed that more than one E2 is most likely involved. Based on my results,

Rad6, Cdc34, and Ubc4 have the most similar phenotypic profile to Rkr1, indicating that they likely fall in a similar pathway. However, loss of *RKR1* causes an increase in nonstop protein levels of a reporter construct while no E2 mutant exhibited the same phenotype. This indicates that there is most likely redundancy in a Rkr1-E2 partnership. I was also unable to test the essential E2 Ubc1, and so this protein is still a viable candidate for this interaction. Interestingly, a large-scale screen using human RING domains and human E2s in a yeast two-hybrid assay identified an interaction between human Rkr1 and Ubc4/5 (VAN WIJK *et al.* 2009). Although this result was not confirmed with purified proteins, it may be validated as additional studies are performed to expand the network of E2-E3 interactions in other organisms.

Lastly, I investigated Htz1 as a substrate for Rkr1 ubiquitylation. Collaboration with Dr. Nevan Krogan produced an E-MAP analysis of *rkr1Δ* cells, which identifies mutations with similar genetic profiles that most likely function in similar pathways. Strains deleted of *RKR1* exhibit a genetic profile similar that of strains lacking the histone variant Htz1 or members of the Swr1 complex, which functions to exchange Htz1 for canonical H2A in nucleosomes (KROGAN *et al.* 2004; KROGAN *et al.* 2003a; MIZUGUCHI *et al.* 2004). Preliminary studies by Mary Braun and Elia Crisucci found that Htz1 was a target for Rkr1-mediated ubiquitylation when *HTZ1* was overexpressed. I expanded on these studies, showing that endogenous Htz1 is not targeted for ubiquitylation. This suggested that Htz1 was ubiquitylated only when overexpressed and therefore was a substrate of quality control. Further, I showed that an HA-tagged Htz1 was not a substrate for Rkr1 when overexpressed, unlike the FLAG-tagged Htz1 construct. Rkr1 may therefore be targeting this FLAG tag, which contains multiple lysine residues similar to the poly-lysine chain on nonstop proteins. However, in order to further clarify this result, it would be interesting to test other overexpressed FLAG-tagged substrates for Rkr1-mediated ubiquitylation.

To date, Rkr1 has not been shown to ubiquitylate proteins other than nonstop substrates. My results from this thesis support the role for Rkr1 in protein quality control, and it is not unlikely that Rkr1 also recognizes as yet unidentified targets outside of nonstop.

5.3 FINAL CONCLUSIONS

My thesis work has aimed to understand the connection between Rtf1 and Rkr1, as well as to identify additional roles for these proteins. In my studies, I have found support for the role of Rkr1 in quality control of aberrant proteins. Importantly, I have also shown that the prion [*PSI*$^{+}$] negatively influences the loss of *RKR1* and adds to a burden in quality control (Figure 32). I have shown that this is lethal in the absence of *RTF1*, although the mechanism behind this synthetic lethality is still unclear and remains an avenue for future studies. Loss of *RTF1* and H2B ubiquitylation may increase problems in quality control by affecting mRNA processing and creating additional mRNA substrates, resulting in a lethal stress to [*PSI*$^{+}$] *rkr1Δ* cells. In an alternative hypothesis, loss of *RTF1* could cause a change in products needed to protect cells from quality control in [*PSI*$^{+}$] *rkr1Δ* conditions. Future studies into the effect of *rtf1Δ* on transcript levels and processing may clarify these ideas.

In addition, my genetic suppressor results leave two interesting paths for further study of Rtf1 and Rkr1. Through Chl1 and Rpc17, I have potentially connected these factors to sister chromatid cohesion and translation in previously unappreciated roles. Therefore, future studies in these areas may reveal exciting new roles for Rtf1 and Rkr1, and, in addition, for Chl1 and Rpc17 (Figure 32).

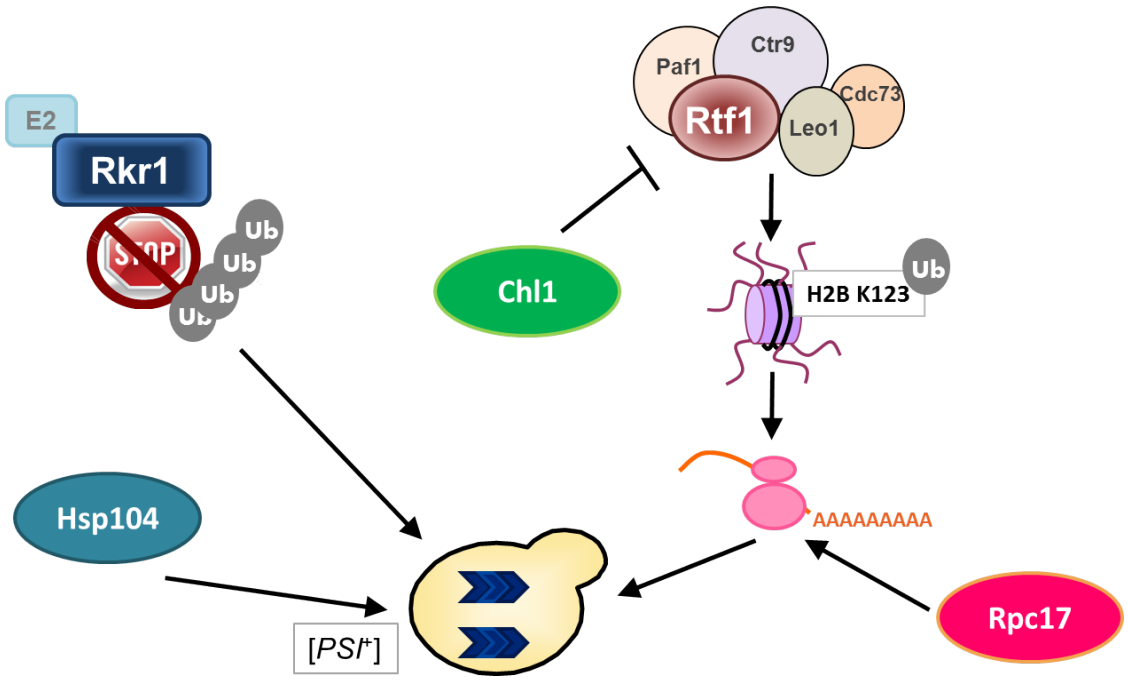

Figure 32: Final conclusions on suppression mechanisms of *rtf1Δ rkr1Δ* synthetic lethality

Through a transposon mutagenesis screen, I identified mutations in the genes encoding Hsp104, Chl1, and Rpc17 that rescue *rtf1Δ rkr1Δ* synthetic lethality. Hsp104 inactivation or overexpression clears yeast of the prion [*PSI*$^{+}$], which increases quality control problems in yeast, both in protein and aberrant mRNA. Therefore, it may be that Rtf1, as a transcription factor, is needed to protect yeast in the face of this increased burden. The suppression mechanism of a *chl1Δ* mutation is unclear, but loss of *CHL1* opposes *rtf1Δ* -specific defects in nonsense and nonstop products. Depletion of Rtf1 may therefore cause changes in RNA processing that lead to an additional accumulation in aberrant mRNAs and deletion of Chl1 may suppress these errors. It remains to be investigated if the role of Chl1 in sister chromatid cohesion is important in this suppression mechanism, or if Chl1 also plays a role in transcription. Finally, I've shown that a mutation in the essential gene *RPC17* is also a genetic suppressor of *rtf1Δ rkr1Δ* synthetic lethality. One hypothesis to explain this suppression is that this mutation affects RNA Pol III function and tRNA levels, with downstream effects on translation efficiency and the accumulation of quality control products.

6.0 APPENDIX

ADDITIONAL STUDIES INTO THE FUNCTIONS OF RKR1 AND THE PAF1 COMPLEX

6.1 PAF1C-DEPENDENT HISTONE MODIFICATIONS ARE NOT SIGNIFICANTLY AFFECTED IN HISTONE ACETYLTRANSFERASE OR HISTONE DEMETHYLASE MUTANT STRAINS

Transcription occurs within the controlled context of chromatin, which may is altered by various mechanisms in order to accommodate elongating RNA polymerase II (Pol II) and transcribe the DNA template (LI *et al.* 2007a). Chromatin alterations include ATP-dependent chromatin remodeling, the exchange of histone variants, and histone modifications such as methylation, ubiquitylation, and acetylation. Histone modifications are patterned specifically to allow the transcription or repression of genes (LI *et al.* 2007a).

Several factors are required to establish the correct pattern of histone modifications necessary for proper transcription. One such set of factors is the Paf1 complex (Paf1c). Rtf1 of Paf1c is necessary for the mono-ubiquitylation of K123 on H2B, which is a prerequisite for downstream methylation of H3 K4 and K79 by Set1 and Dot1, respectively (DOVER *et al.* 2002;

SUN and ALLIS 2002). These specific ubiquitylation and methylation marks are regulated most strongly by Rtf1 of the Paf1 complex, and are associated with active transcription.

Besides these marks, numerous other histone modifications have been characterized. These include methylation of H3 K36 by the methyltransferase Set2 (STRAHL *et al.* 2002) as well as histone acetylation by HATs, which is typically a mark of active transcription (POKHOLOK *et al.* 2005). Interestingly, these two modifications are mechanistically coupled, as H3 K36 di-methylation recruits the Rpd3s HDAC, which deacetylates histones and represses cryptic initiation within genes (CARROZZA *et al.* 2005; KEOGH *et al.* 2005; LI *et al.* 2009). In addition to HDACs, cells also contain histone demethylases (DMTs), which have been shown to demethylate methylated histones (reviewed in (MOSAMMAPARAST and SHI 2010)). This suggests evidence for an extremely dynamic chromatin environment, with multiple players involved in the establishment and erasure of correct histone modifications.

In a study to investigate if Paf1c played a role in histone modification pathways other than the H2B K123 ubiquitylation pathway, western analysis and chromatin immunoprecipitation (ChIP) was used to examine the effect of deleting members of Paf1c on H3 K36 tri-methylation. Deletion of *PAF1* or *CTR9* specifically abolishes H3 K36 tri-methylation and deletion of *CDC73* reduces this mark, while loss of *RTF1* or *LEO* has little effect (CHU *et al.* 2007). This result was specific to this methylation state, as loss of these subunits had little to no effect on di-methylation of H3 K36. Set2 recruitment to active genes is decreased in *paf1Δ* strains, but more severely at the 3' ends than the 5' ends. Interestingly, deletion of *PAF1* also causes an increase in H3 and H4 acetylation at the 5' end of genes (CHU *et al.* 2007). These results suggested that in the absence of Paf1, an increase in acetylation may modulate a corresponding decrease in H3 K36 tri-methylation.

To test this hypothesis, I investigated HATs that might be responsible the for increased histone acetylation observed in a *paf1*Δ strain, as well as DMTs which may be hyperactive at the 5' end of genes in the absence of Paf1 and cause a decrease in H3 K36 tri-methylation. To this end, I first continued genetic analysis started by Rajna Simic to investigate genetic interactions between *paf1Δ* and loss of several yeast HATs or DMTs. A genetic interaction may indicate that these two factors are required for similar processes. I found no genetic interaction between *paf1Δ* and mutations in any of five proposed DMTs (*ECM5*, *GIS1*, *YJR119C*, *RPH1*, and *JHD1*) (KWON and AHN 2011) (Table 7). However, I found that deletion of *PAF1* caused sickness or lethality in combination with loss of the HATs or HAT subunits *GCN5*, *VID21*, *RTT109*, or *SAS2* (Table 8, Figure 33) (LEE and WORKMAN 2007).

Next, I performed western analysis of single HAT or DMT mutant strains, as well as double mutants with *paf1Δ* to identify for strains that exhibited a decrease in histone acetylation or an increase in H3 K36 tri-methylation back to wild type levels. To perform these experiments, I used multiple antibodies against histone methylation and histone acetylation available in the lab, in order to observe any additional or unexpected changes in any histone modification states in the mutant strains. However, I did not detect reversal of H3 K36 tri-methylation or histone acetylation back to wild type levels in any double mutant strain with *paf1Δ* and HATΔ or DMTΔ (Subset of data shown in Figure 33). These results may indicate that multiple pathways are redundantly at work in regulating histone acetylation and H3 K36 tri-methylation with Paf1 (See also Table 7 and 8). For example, both Jhd1 and Rph1 are DMTs for H3 K36 methylation, which is decreased in the absence of *PAF1* (CHU *et al.* 2007; KIM and BURATOWSKI 2007; KWON and AHN 2011). Therefore, I also created a *jhd1Δ rph1Δ paf1Δ* strain but found that it also did not significantly enhance H3 K36 tri-methylation levels (Figure 34).

Loss of *PAF1* results in a loss of H3 K36 tri-methylation and corresponding increase in acetylation of histone H3 and H4 at the 5' ends of genes (CHU *et al.* 2007). Here, I have described experiments that sought a DMT or HAT that may be recruited in the absence of Paf1. However, these preliminary investigations did not identify potential factor(s). This may be due to the nature of western analysis, which reveals the global state of histone modification and may not detect subtle changes observable by ChIP. Redundancy in these histone modification pathways may also have made these experiments difficult to interpret. Lastly, these results may suggest that additional factors are involved in this process. For example, instead of a HAT being recruited to increase histone acetylation at the 5' end of genes in *paf1Δ* cells, there may be a deficiency in HDAC recruitment resulting in the inability to deacetylate histones. The HDAC Set3 has been shown to deacetylates histone at the 5' ends of genes in response to H3 K4 di-methylation (KIM and BURATOWSKI 2009). Similarly, rather than the recruitment of a DMT to demethylate H3 K36 tri-methylation in *paf1Δ* cells, the catalytic activity of Set2 may be impaired, blocking progression of H3 K36 di-methylation to tri-methylation. Interestingly, it has recently been shown that improper phosphorylation of the RNA Pol II C-terminal domain results in global loss of H3 K36 tri-methylation, although this also correlates with a decrease in Set2 levels (FUCHS *et al.* 2012). Future studies will likely illuminate how these factors and others contribute to such a complex set of histone modifications necessary for proper transcription.

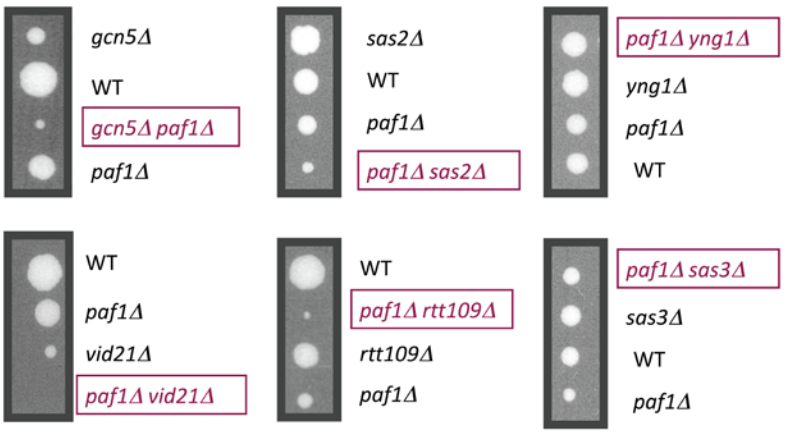

Figure 33: Genetic interactions between *paf1Δ* and histone acetyltransferase mutants.

Tetrad dissections of diploids that were heterozygous for *paf1Δ* and a deletion of a histone acetyltransferase protein (Crosses performed by Rajna Simjic and scored by Kristin Klucevsek). Shown is one representative tetrad from the dissection plate.

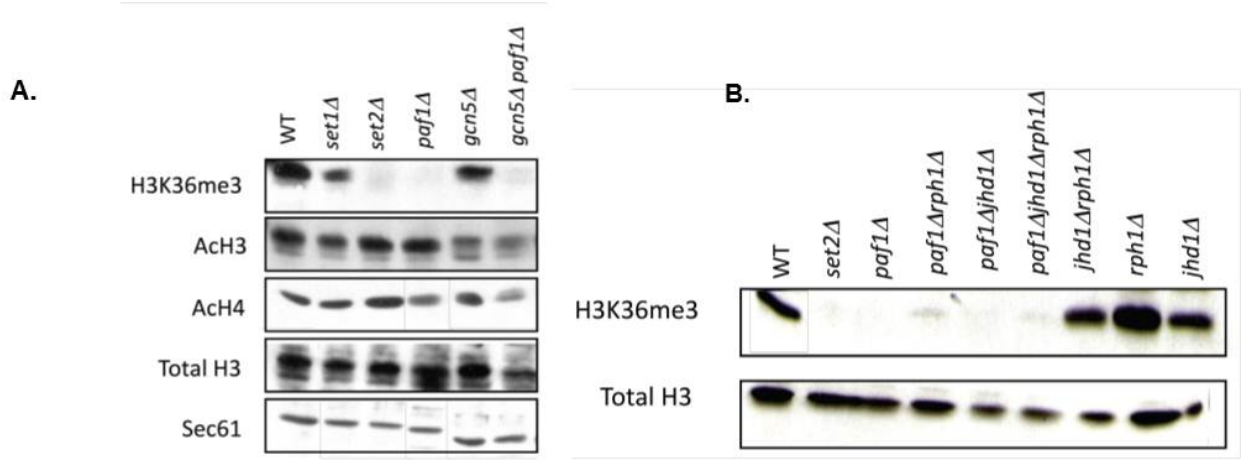

Figure 34: Western analysis of histone modifications in strains lacking the HAT Gcn5 or various DMTs.

(A). Wild type (KY311), *set1Δ (*KY941), *set2Δ* (KY914), *dot1Δ* (KY935), *paf1Δ* (KY686), *gcn5Δ* (KY1343), or *gcn5Δ paf1Δ* (KY1347) extracts were made using RIPA buffer and assayed for histone modifications using western analysis and standard techniques. Antibodies used were against H3 acetylation (AcH3, 1:2000; Upstate), H4 acetylation (AcH4, 1:40,000; Upstate), Sec61 (1:1000; Gift from Dr. Jeff Brodsky), total H3 (1:2000, Abcam), and H3 K36me3 (1:3000, Abcam). (B). Wild type (KY311), *set2Δ* (KY914), *paf1Δ* (KY686), *paf1Δ rph1Δ* (KY1339), *paf1Δ jhd1Δ* (KY1334), *paf1Δ jhd1Δ rph1Δ* (KY1393), jhd1Δ *rph1Δ* (KY1396), *rph1Δ* (KY1337), or *jhd1Δ* (KY1333) extracts were made and analyzed as in (A) with antibodies against H3 K36 tri-methylation or total H3 levels as a control.

Table 7: HAT or HAT complex mutations used in Appendix

(*) Indicates subunit of HAT complex that is not known to possess HAT activity but is an important part of the complex. Red indicates acetylation recognized by AcH3 or AcH4 antibodies.

HAT or HAT complex subunit	Complex	Histone Substrate(s)
Gcn5	SAGA/SLIK, ADA	H2B, H3K9,14,18,23, 16, Htz1K14
Sas2	SAS	H4K16
Sas3	NuA3	H3K14, 23
Yng1*	NuA3	H3K14, 23
Vid21*	NuA4	H2AK4,7 H4K5, 8,12,16
Rtt109		H3K9, 56

Table 8: DMT proteins studied in Appendix

DMT	Histone Substrate
Jhd1	H3K36me2
Jhd2	H3K4me2,3
Rph1	H3K36me2,3
Gis1	Predicted to have no catalytic activity
Ecm5	Predicted to have no catalytic activity

6.2 A SCREEN TO IDENTIFY HISTONE H3 OR H4 RESIDUES THAT GENETICALLY OR FUNCTIONALLY INTERACT WITH LOSS OF *RKR1*

Rkr1 was initially identified as a protein that is required in the absence of the transcription factor Rtf1 for cell viability, as well the Rtf1-mediated histone modification, H2B K123 monoubiquitylation (BRAUN *et al.* 2007; NG *et al.* 2003a). This is a mark generally associated with active transcription, indicating that Rkr1 might also play a role parallel to this process (XIAO *et al.* 2005). Importantly, H2B ubiquitylation is required for downstream methylation of histone H3 on lysines 4 and 36, which is also a mark of active transcription (DOVER *et al.* 2002; SUN and ALLIS 2002; WOOD *et al.* 2003b). Therefore, it was hypothesized that Rkr1 might also have an important role in transcription.

To better understand the function of Rkr1, I performed a synthetic genetic array (SGA) analysis to identify additional histone residues that when mutated caused synthetic lethality in the *rkr1Δ* strains. The Boeke lab has generated a set of integrated histone H3 and H4 point mutations representing 486 alleles where each residue has been systematically changed to an alanine, and each alanine to a serine (DAI *et al.* 2008). Additional amino acid substitutions designed to mimic modification states, such as mutation of all lysine residues to arginine and glutamine to mimic deacetylation or acetylation, respectively (DAI *et al.* 2008). Deletions of different sizes were also made of the histone tails (DAI *et al.* 2008). The collection exists in a 96-well plate format, allowing for easy SGA analysis by performing a cross between these strains and a *rkr1Δ* strain (KMKY81) to identify genetic interactions. Both strains used in this query had only one copy of the histone H3 and H4 genes, with the other endogenous copy deleted.

A genetic screen using these mutants, performed in duplicate, identified 8 mutations that were synthetically lethal or sick in the absence of *RKR1*. They included the following: H3E73A,

H4H18A, H4K16Q, H4K16A, H4Δ13-20, H4Δ17-20, H4Δ9-16, and H4Δ15-18. In order to confirm these interactions, I performed individual crosses with these strains. However, I found that upon using standard techniques to do these crosses, each of these strains actually had difficulties mating with my *rkr1Δ* strain or the resulting diploid had difficulties sporulating. None of these residues had any discernible genetic interactions when tetrad dissections were able to be performed. Therefore, each of the genetic interactions from the original screen were most likely artifacts of the mutant histone strains' inabilities to properly mate or sporulate, which could indicate an important function for these histone residues, but not a function relevant to Rkr1.

Although *RKR1* did not appear to interact genetically with any histone H3 or H4 mutations, I next decided to screen this library for sensitivity to cycloheximide. Strains lacking *RKR1*, as well as strains defective in the H2B ubiquitylation pathway, including strains lacking *RTF1*, *BRE1*, *RAD6*, or containing a H2B K123 substitution, are all sensitive to media cycloheximide. These results indicate that cycloheximide sensitivity might be useful for identifying genes or residues important for a similar process, such as transcription. Therefore, I used a large-scale analysis to first identify histone H3 and H4 mutant strains that appeared to have slow growth on cycloheximide-containing medium. Next, I struck these strains out in duplicate to confirm their poor growth compared to wild type and *rkr1*Δ control cells. Interestingly, I found a specific set of residues that are important for cycloheximide resistance with many of them having established roles in active transcription (DU and BRIGGS 2010; HAINER and MARTENS 2011; POKHOLOK *et al.* 2005; PSATHAS *et al.* 2009) (Table 9). In addition, these residues lie mostly in regions of histone H3 or H4 that are in contact with DNA, indicating that they could be disrupting DNA-histone interactions (Figure 35). Therefore, although I did not

identify any histone H3 or H4 residues that are synthetically lethal in the context of a *rkr1Δ* strain, I did identify histone amino acid substitutions that share an important phenotype with *rkr1Δ* and H2B K123R, further suggesting that this phenotype may be indicative of an important cellular process connected to transcription.

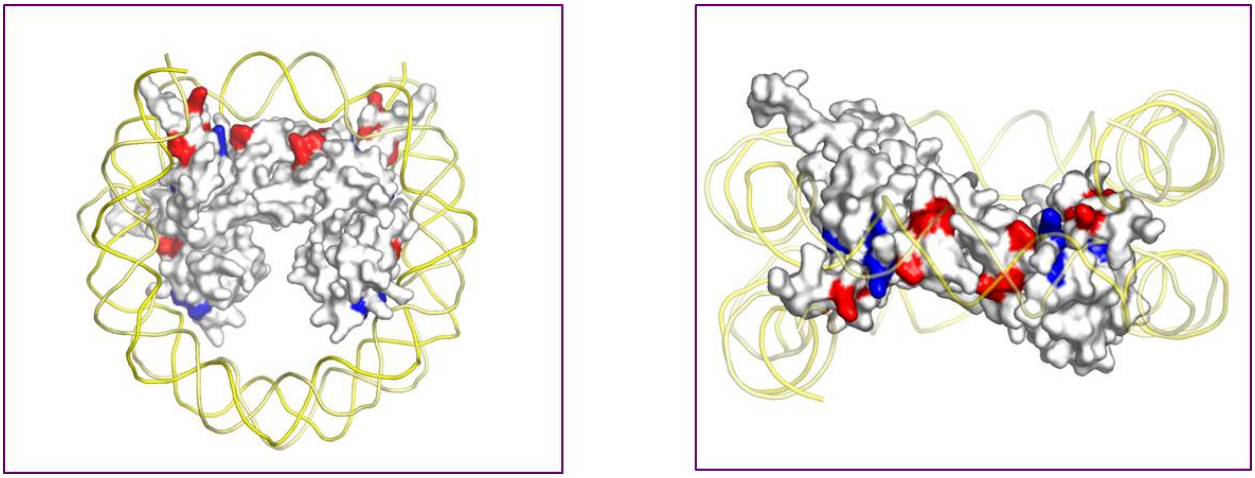

Figure 35: Residues that are required for cycloheximide resistance.

Histone H3 (red) and Histone H4 (blue) residues identified in a screen for mutants sensitive to cycloheximide (See table 9). The positions of the amino acids are shown on the structure of histone H3 and H4, as well as their contact with DNA (LUGER *et al.* 1997).

Table 9: Residues that are required for cycloheximide resistance

Strain	CHX^R	Modification/Function
WT	+	
rkr1Δ	--	
H3Δ1-12	---	Tail Modifications
H3Δ13-28	---	Tail Modifications
H3Δ33-36	---	Tail Modifications
H3K4,9,14,16A	--	A; Active Txn
H3K4,9,14,16R	--	A; Active Txn
H3K4,9,14,16Q	+	A; Active Txn
H3K4A	-	M; Active Txn
H3Q5A	---	
H3K36A	-	M; Active Txn
H3K42A	---	
H3K42Q	-	
H3G44A	---	
H3R49A	--	
H3Q68A	--	

Strain	CHX^R	Modification/Function
H3I89A	---	
H3V117A	-	Affects K36me^3, Cryptic Txn
H3Q120A	---	Cryptic Txn
H3K122A	--	A; Humans
H3K122Q	-	A; Humans
H4Δ17-24	-	Tail Modifications
H4R36A	---	
H4K44A	--	
H4I46A	-	
H4R78K	---	
H4T80A	---	P; Active Txn and Cell Division
H4R78K	---	

A = Acetylation
P = Phosphorylation
M = Methylation

CHX^R	CHX^S
+ -	-- ---

6.3 THE EFFECT OF RKR1 ON CELL CYCLE PROGRESSION

A screen was performed to identify mutations that were synthetically lethal in the absence of the Paflc member, Rtf1 (COSTA and ARNDT 2000). Nine genes were found in this screen, which represented a range of cellular functions, with 8 of the 9 having either direct or indirect connections to transcription (Table 10) (Costa and Arndt, unpublished data and (COSTA and ARNDT 2000)). There was one gene, which was later named *RKR1*, which coded for an uncharacterized open reading frame that has since been identified as a ubiquitin ligase involved in nonstop protein degradation (BENGTSON and JOAZEIRO 2010; BRAUN *et al.* 2007; WILSON *et al.* 2007). Because most of the genes recovered from the synthetic lethality screen with *rtf1Δ* were involved in transcription, we hypothesized that Rkr1 might also have connections to the cellular processes represented by these results.

To help in uncovering the function of Rkr1, a phenotypic screen was performed to identify conditions which required Rkr1 function (See Chapter 4). The most notable phenotype that resulted from this screen was a sensitivity to cycloheximide. Although the cause of this uncharacterized phenotype is not clear, cycloheximide interrupts translation and has been linked to increases in aberrant mRNA low levels of ubiquitin or cell cycle disruptions (FRISCHMEYER *et al.* 2002; HANNA *et al.* 2003; MCCUSKER and HABER 1988; WAGNER and LYKKE-ANDERSEN 2002). Interestingly, two other genes encoding mutations that are synthetically lethal with *rtf1Δ* are *SWI4* and *SWI6*, which are transcriptional activators needed for proper cell cycle regulation (NASMYTH and DIRICK 1991). The cause of their synthetic lethality with *rtf1Δ* is currently unknown. However, I also wanted to know if Rkr1 was involved in proper regulation of the cell cycle. To investigate this, I performed a cell cycle arrest of wild type or *rkr1Δ* cells. As a control, *swi4Δ* cells were used. Briefly, mating type **a** cells were grown to an OD_{600} of 0.2, treated with 5

μg/ml α-factor, and incubated at 30°C for 2.5 hours or until cell cycle arrest in G1 phase was observed. Cells were transferred to a new sterile tube and released from α-factor by washing and resuspending in fresh YPD. Cultures were then allowed to continue growing while samples were taken at the indicated time points. These samples were sonicated to separate individual cells. The number of unbudded, small budded, and large budded cells were counted. This experiment was performed in duplicate using blind analysis, without knowing which samples represented which strain.

As expected, the loss of *SWI4* resulted in an S to G2 cell cycle delay compared to wild type cells (Figure 36). However, the loss of *RKR1* had no discernible effect on the cell cycle in any phase (Figure 36). Therefore, Rkr1 is not required for proper cell cycle progression and its genetic interaction with *rtf1Δ* or its cycloheximide phenotype is not likely due to a cell cycle defect.

Table 10: Genes uncovered from *rtf1Δ* synthetic lethality screen

Gene	Function
SWI6	Transcriptional activator
SWI4	Transcriptional activator
PLC1	Signal transduction
ARG82	Signal transduction
SRB5	Srb/Mediator component
CTK1	Pol II CTD kinase
FCP1	Pol II CTD phosphatase
POB3	FACT, elongation factor
YMR247c/RKR1	Nonstop Protein Quality Control

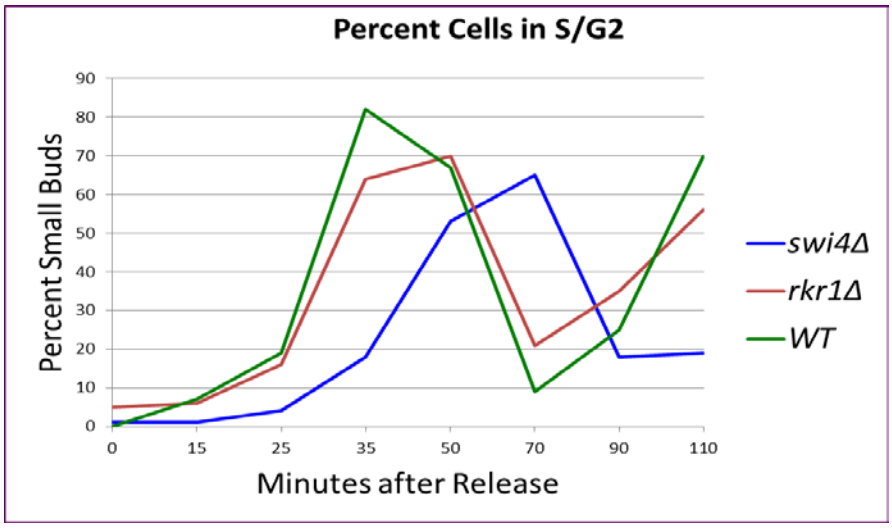

Figure 36: *rkr1*Δ strains do not have a cell cycle delay

Wild type (KY669), *rkr1*Δ (MBY168), or *swi4*Δ (KK10) strains were grown in YPD to log phase and arrested with α-factor. Cells were released by washing in YPD and allowed to grow at 30°C. The number of cells unbudded (G1 to S phase), with small buds (G2 phase), or large buds (M phase), were counted at the indicated time points. Shown are the percent of small budded cells, which represents cells transitioning from S into G2. No differences were seen in the numbers of unbudded or large budded cells compared between any of the strains (data not shown). The Experiment was performed in duplicate, with one representative experiment shown.

Table 11: *S. cerevisiae* strains used in Appendix

Strain	Genotype
KKY10	*MAT* a *swi4Δ::HIS3 lyst-128δ leu2Δ1 ura3-52 trp1Δ1*
MBY39	*MAT* a *rkr1Δ::KanMX4 his3Δ200 lys2-128δ leu2Δ1 trp1Δ63*
KMKY81	*MAT* α *rkr1Δ::KanMX ht1-hhf1::NatMX4 CAN1::MFA1pr-HIS3 his3Δ200 leu2Δ1/0 ura3-52/0 trp1Δ63 lys2Δ0*
KY311	*MAT* α *his3Δ200 leu2Δ1 ura3-52*
KY686	*MAT* a *paf1Δ::URA3 ura3-52 lys2-128δ*
KY669	*MAT* a *his3Δ200 lys2-128δ leu2Δ1 ura3-52 trp1Δ63*
KY914	*MAT* α *set2Δ::HIS3 his3Δ200 lys2-173R2 leu2Δ1 ura3-52*
KY935	*MAT* a *dot1Δ::HIS3 his3Δ200 ura3-52*
KY941	*MAT* α *set1Δ::HIS3 his3Δ200 ura3-52*
KY1333	*MAT* a *jhd1Δ::KanMX his3Δ200 leu2Δ1 ura3-52 lys2-173R2*
KY1334	*MAT* α *jhd1Δ::KanMX paf1Δ::URA3 his3Δ200 leu2Δ1 ura3-52*
KY1337	*MAT* a *rph1 his3Δ200 ura3-52 trp1Δ63*
KY1339	*MAT* a *rph1Δ::KanMX paf1Δ::URA3 his3Δ200 ura3-52 trp1Δ63*
KY1343	*MAT* α *gcn5Δ::HIS3 his3Δ200 leu2Δ1 ura3-52 trp1Δ63*
KY1347	*MAT* a *gcn5Δ::HIS3 paf1Δ::URA3 his3Δ200 leu2Δ1 ura3-52*
KY1393	*MAT* a *jhd1Δ::KanMX paf1Δ::URA3 rph1Δ::KanMX his3Δ200 ura3-52*
KY1396	*MAT* α *jhd1Δ::KanMX rph1Δ::KanMX his3Δ200 ura3-52 arg4-12*

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