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The RNA binding protein Nab2 genetically interacts with multiple RNA exosome cofactors to regulate target RNAs

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Abstract

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Christy E. Kinney

RNA binding proteins play important roles in the processing and precise regulation of RNAs. Highlighting the biological importance of RNA binding proteins is the increasing number of human diseases that result from mutations in genes that encode these proteins. We recently discovered that mutations in the *ZC3H14* gene, which encodes an evolutionarily conserved poly(A) tail RNA-binding protein, cause intellectual disability. The majority of studies that have provided insight into the function of ZC3H14 have exploited the budding yeast model to study the ZC3H14 orthologue, Nab2. The *NAB2* gene is essential in *S. cerevisiae* and conditional *nab2* mutants cause defects in a number of RNA processing steps. To explore the critical functions of the Nab2/ZC3H14 protein family, we performed a high-copy suppressor screen on *nab2* mutant yeast cells. This screen identified genes encoding two core subunits of the RNA exosome, Rrp41 and Rrp42, as well as Nrd1 and Ski7, nuclear and cytoplasmic cofactors of the RNA exosome, respectively. We also identified nucleolar cofactor Nop8 and nuclear cofactor Mtr4 as suppressors; however, we focused this study on elucidating the genetic interactions between Nab2 and cofactors Nrd1 and Ski7. Using structure function analysis, we determined that the RNA binding function of Nrd1 is required for the suppression of growth defects in *nab2* mutant cells, while the RNA exosome-interacting domain is required for Ski7-mediated suppression. In conjunction with previous data, our results support a model for RNA exosome impairment through overexpression of cofactors. We also used RNA-seq analysis to identify transcriptomic effects of overexpression of suppressors in *nab2* mutant cells. We first compared the transcriptome of *nab2* mutant to control cells and identified significantly affected transcripts and gene ontology categories. We next examined the effects of overexpression of suppressors in *nab2* mutant cells and revealed distinct transcriptomes with broad but subtle effects on RNAs. These results are consistent with a global role of Nab2 in modulating transcript stability. This study uncovers functional interactions between the RNA exosome and Nab2 in both the nucleus and the cytoplasm.

The RNA binding protein Nab2 genetically interacts with multiple RNA exosome cofactors to regulate target RNAs

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Chapter 1:

General introduction

1.1 The many classes of RNA

The central dogma of molecular biology states that DNA is transcribed into RNA which is then translated into proteins. Thinking about this dogma is much like taking a plane ride from New York to Los Angeles. The trip begins and ends in exciting, bustling places, but the plane must still traverse the length of the country to reach its destination. RNA, like “flyover country”, is often the dismissed or forgotten heartland.

There are many different classes of RNA. Although the Central Dogma mentions RNA as an intermediate step to proteins, there are multiple classes of RNAs that are not translated into proteins, instead playing regulatory roles. Messenger RNAs (mRNAs), or coding RNAs, are RNAs destined for translation. Regulatory non-coding RNA (ncRNA) classes include, just to name a few of this ever expanding class, small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), and long non-coding RNAs (lncRNAs) [1]. Additional classes of RNAs whose functions are still under investigation include cryptic unstable transcripts (CUTs), stable uncharacterized transcripts (SUTs), and Nrd1-unterminated transcripts (NUTs) [2, 3]. There are also RNAs that play structural roles, such as the RNA component of the telomerase complex, TLC1 [4]. With such an array of RNA classes, proper transcription and post-transcriptional regulation is key to ensure proper function and regulation.

1.2 The budding yeast model system is used to study evolutionarily conserved RNA processing steps

The many steps of RNA regulation and processing are highly conserved across species. Due to this conservation, model systems have been critical in characterizing

these processes and elucidating the interplay of so many critical aspects of RNA processing. The *S. cerevisiae* budding yeast model system has been particularly instrumental in defining RNA processing steps. This eukaryotic model system has a rapid reproduction cycle, a simple genome, and is extremely amenable to genetic manipulation [5]. Previous studies have described processing steps in budding yeast, such as mRNA splicing [6], rRNA processing [7], and RNA turnover [8], and current studies continue to take advantage of this model system for further characterization of conserved RNA processing steps.

1.3 RNA binding proteins play pivotal regulatory roles across species

Regulation of RNAs, including post-transcriptional control of gene expression, is vital to biological function. The co- and post-transcriptional processing of transcripts, the degradation of aberrantly transcribed RNAs, and the control of translation are among critical regulatory roles played by an important class of proteins called RNA binding proteins (RBPs) [9]. The roles played by RBPs, as well as their regulatory effects on target RNAs, are increasingly being studied as their connections to organismal function as well as human disease are discovered [10].

1.4 RNA binding proteins in plants

Highlighting the importance of RNA binding proteins are the pivotal roles they play in typical plant function and the adaptation of plants to the effects of climate change. In *Arabidopsis thaliana*, RBPs play important roles in controlling flowering time [11], salt-stress tolerance [12], and responses to plant hormones [13]. RBPs are also

rising to the forefront as regulators of processes conferring tolerance to various climate effects, including drought-tolerance in rice [14], drought-tolerance in wheat [15], and cold-tolerance in rice [16], among other agronomic concerns. Uncovering roles of additional RBPs in plant adaptation may be a major key to feeding an expanding human population in an increasingly unstable global climate.

1.5 RNA binding proteins are implicated in many human diseases

RNA binding proteins are also being identified as key factors in an expanding number of human diseases. These diseases include various cancers [17], diabetes [18], muscular atrophy disorders, and neurodegenerative disorders. Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's Disease, is characterized by degeneration of motor neurons. Many inter-related molecular processes, such as stress granule formation and nucleocytoplasmic shuffling, are implicated in ALS, and these processes involve dozens of RNA binding proteins [19]. Huntington's disease is a neurodegenerative disorder characterized by the degeneration of neurons, and RNA binding proteins control the variable expression of the huntingtin (*HTT*) gene, contributing to the disease phenotype [20, 21]. RNA binding proteins are also implicated in Parkinson's disease and Alzheimer's disease [22].

A major conundrum is that many of the RBPs linked to human disease are ubiquitously expressed, but the clinical manifestations are specific to certain tissues and cell types. Furthermore, RBPs often play many different roles and partner with different proteins to confer function. Thus, to understand how mutations in genes encoding RBPs contribute to human disease, it is critical to both define the functions of that protein,

which are often multi-faceted, and consider those function in the context of the tissue impacted. Such approaches require detailed analysis of individual RBPs to understand complex functions and functional context.

1.6 ZC3H14 is implicated in a non-syndromic form of intellectual disability

ZC3H14 is a ubiquitously expressed RNA binding protein. Loss of function mutations in the *ZC3H14* gene cause a non-syndromic form of autosomal recessive intellectual disability (NS-ARID), and affected individuals have low I.Q. scores (~30-50) compared to ~100 as the average score [23]. One identified *ZC3H14* disease-causing mutation is homozygous *R154X*, which causes a premature stop codon in exon 6 and a loss of all nuclear ZC3H14 [23]. As ZC3H14 is ubiquitously expressed, the NS-ARID phenotype is striking. To understand how mutations in *ZC3H14* can cause a brain-specific phenotype, it is imperative to understand how ZC3H14 functions, including roles in regulation of gene expression, protein-protein interactions, and RNA targets of this RNA binding protein. Thus far, studies of human ZC3H14 have revealed important roles in modulating poly(A) tail length [24], splicing in neuronal cells, a role in quality control and RNA export [25], and a role in neuroprotection [26]. However, which, if any, of these functions are critical for proper neurological function is not yet clear.

1.7 ZC3H14 is a highly conserved RNA binding protein

ZC3H14 is evolutionarily conserved from humans to *S. cerevisiae*. Human, mouse, fly, and yeast orthologs share the N-terminal PWI (Proline-Tryptophan-Isoleucine) domain critical for protein-protein interactions, a nuclear localization signal

domain, and a C-terminal Cys_3His Zinc finger RNA binding domain [27-29] (Figure1-1). Model systems have been heavily employed to determine functional conservation, discover additional cellular roles of ZC3H14, and provide insight into the mechanisms underlying the intellectual disability phenotype.

The mouse and *Drosophila* orthologs, conserved as ZC3H14 and Nab2, respectively, have been used to understand the neurological impact of the mutated gene and loss of protein function. Recent studies in *Drosophila* have uncovered the roles of Nab2 in locomotion, development [23], neuronal morphology, and neuronal function [23, 30], as well as the role of Nab2 in brain development and working memory [31]. Furthermore, an interaction of Nab2 and the Fragile X protein ortholog FMRP has been identified [32]. Additionally, Nab2 regulates m^6A mediated splicing of the *Sex-lethal* gene in *Drosophila*, supporting a role for Nab2 in RNA splicing as well as a mechanism for target specificity [33]. In mice, analysis of ZC3H14 has revealed a role in working memory, regulation of poly(A) tail length, dendritic spine density and morphology, and composition of synaptosomes [34, 35]. Mouse ZC3H14 is also involved in neuronal transcript processing and splicing [36, 37]. Altogether, these findings provide insight into the neuronal functions of ZC3H14 and establish potential mechanisms for how *ZC3H14* mutations may lead to brain-specific disease.

1.8 *Nab2* mutants in *S. cerevisiae* are used to provide insight into potential functions of ZC3H14

The budding yeast orthologue of the human ZC3H14 protein is the nuclear poly-adenylated RNA binding protein 2 (Nab2) [38, 39]. Previous work identified roles for

Nab2 in RNA quality control, poly(A) tail length control, nuclear export, and stability of target RNAs [40-45]. In humans, *ZC3H14* is not essential for viability, as loss of function mutations result in intellectual disability rather than loss of viability [23]. In *S. cerevisiae*, however, the *NAB2* gene is essential, and conditional mutants of *NAB2* are, therefore, used to study Nab2. The generation of Nab2 variants with mutations in specific domains has allowed focused study of Nab2 functions and interactions [27]. Genetic and functional analyses on conserved domains shared between ZC3H14 and Nab2 in *S. cerevisiae* have assisted in uncovering roles and interactions of ZC3H14 and continue to provide insight into how loss of ZC3H14 function may contribute to pathology in humans [46].

1.9 *Nab2-C437S* is a cold-sensitive mutant

One *S. cerevisiae nab2* mutant, *nab2-C437S* [47], carries a cysteine to serine missense mutation in the sixth Cys3His zinc finger of the zinc-finger RNA-binding domain, decreasing binding between Nab2 and poly(A) tracts (Cy3 tagged-A_{25}) by approximately 4-fold [28]. *nab2-C437S* mutant cells show an increase in bulk poly(A)-tail length but lack the poly(A) RNA export defect exhibited by other *nab2* mutants [48, 49]. The *nab2-C437S* mutant was originally identified through a random mutagenesis screen seeking conditional mutants of *NAB2*. Yeast cells that express Nab2-C437S as the sole copy of Nab2 display a cold-sensitive growth defect at 25°C or below [47]. As the *NAB2* gene is essential [39], a conditional mutant such as *nab2-C437S* can be invaluable to explore the critical requirement for Nab2 *in vivo*.

Another point of consideration is how the *nab2-C437S* mutation results in a cold-sensitive growth phenotype. Generally, why certain mutations cause cold-sensitive cell growth is not known [50]. In some specific cases, such mutations can lead to stabilization of interactions that need to be dynamic such as some of the Bad Response to Refrigeration (*BRR*) genes identified in a screen for RNA splicing defects [51]. However, why this particular change in Nab2 would confer cold-sensitive growth is not clear. One possibility is that Nab2 needs to be removed from the mature mRNA at a specific point. A genetic interaction with the nuclear pore-localized RNA helicase, Dbp5, might support this hypothesis [47]. Regardless of the mechanism, the cold-sensitive *nab2-C437S* cells provides a conditional mutant of this essential gene.

1.10 Characterizing *nab2-C437S* using a high-copy suppressor screen

To gain insight into what functions of Nab2 are most critical in the cell, a high-copy suppressor screen was performed on *nab2-C437S* cells. This screen utilized a 2μ overexpression library of the entire yeast genome. Plasmids were transformed into *nab2-C437S* cells, and cells were grown at 25°C to test for genes that could suppress the cold-sensitive growth phenotype of *nab2-C437S* cells.

When performing a genetic screen such as the high copy suppressor screen performed here, several points need to be taken into consideration when contemplating how suppressors could link to the starting mutant of interest [5]. Suppressors can rescue growth through direct or indirect mechanisms and interactions. The proteins encoded by the suppressor genes may interact physically with Nab2 or interact with proteins that interact with Nab2. Suppressors may also regulate the expression of other

genes able to mitigate the cold-sensitive growth defect. All of these considerations need to be taken into account in analyzing the suppressors identified and exploring the functional consequences of these interactions.

1.11 RNA exosome subunits and cofactors are high-copy suppressors of *nab2-C437S*

Identified in the high-copy suppressor screen were genes encoding multiple subunits and cofactors of an essential ribonuclease protein complex called the RNA exosome. The ten-subunit RNA exosome has both endonucleolytic and exonucleolytic roles and interacts with cofactors to target various classes of RNAs for degradation or processing [52-56]. The complex consists of three cap subunits and six structural subunits that form a cylindrical barrel through which RNAs are threaded (Figure 1-2). At the bottom of the complex is Dis3, a catalytic subunit responsible for the endo- and exonucleolytic activities [57, 58]. Rrp6 is an exonuclease that functions as an additional nuclear RNA exosome subunit, sometimes being labeled as a cofactor, and it is the only subunit that is not essential [59, 60]. Although the RNA exosome localizes to both the nucleus and the cytoplasm, it is required in the nucleus where it forms the basis of the primary degradation pathway. In the cytoplasm, the Xrn1 degradation pathway is the primary pathway, providing some redundancy of function with the RNA exosome [61].

The RNA exosome targets many classes of RNAs within many cellular contexts. It is involved in the processing or degradation of almost all classes of RNAs. The complex is engaged in multiple mRNA quality control pathways in the cytoplasm [62], including no-go decay (NGD) [63, 64], nonsense-mediated decay (NMD) [65, 66], and

non-stop decay (NSD) [67]. In the nucleolus, the exosome is critical for precise processing to produce mature rRNAs [68]. In the nucleus, the exosome assists in processing of various pre-RNAs, as well as degradation of aberrantly transcribed or processed RNAs [69].

To specify which RNAs to target and in which contexts, the RNA exosome interacts with cofactors and cofactor complexes. Cofactors are found in the nucleus, nucleolus, and cytoplasm and often work exclusively in one of these compartments. Cofactor levels may change according to cellular needs to guide the RNA exosome to misprocessed transcripts, over-abundant transcripts, or transcripts in need of typical refinement prior to export and translation. Uncovering how cofactors interact with the RNA exosome provides keener insight into how the exosome functions and processes or degrades RNAs.

1.12 The RNA exosome subunits and cofactors are highly conserved

Subunits of the RNA exosome are highly conserved from archaea to humans. Rrp41, Rrp42, and Rrp45, core structural subunits of the exosome, comprise the archaeal exosome [70]. All subunits of the *S. cerevisiae* RNA exosome are conserved in humans as EXOSC1-10 and DIS3 [71, 72]. Many functions of the RNA exosome are shared among species.

RNA exosome cofactors assist in targeting the complex to particular transcripts in specific contexts. Conservation of cofactors from *S. cerevisiae* to humans is a current area of exploration. The budding yeast nuclear Nrd1-Nab3-Sen1 (NNS) complex [73], the nuclear Trf4/5-Air1/2-Mtr4 (TRAMP) complex [74, 75], and the cytoplasmic SKI

complex [76, 77] are at least partially conserved in humans. Human sequence homologs of the components of the NNS complex, for example, have been identified as SCAF4/8 (Nrd1), RALY (Nab3), and SETX (Sen1), but functional relationships are still under investigation [78-81]. Studies of cofactors in human cell lines and model systems continue to identify additional conserved cofactors, establish conserved functions in previously identified cofactors, and elucidate functions that are coordinated through different mechanisms across organisms. These findings produce insight into critical cellular processes and provide information that could eventually be used to develop treatments for human diseases.

1.13 The RNA exosome and cofactors are implicated in multiple human diseases

With the plethora of critical roles and functions of the RNA exosome, multiple diseases have been identified that result from mutations in RNA exosome subunit genes [82]. Mutations in *EXOSC3* and *EXOSC8* are implicated in pontocerebellar hypoplasia, and mutations in *EXOSC2* cause a novel syndrome SHRF (short stature, hearing loss, retinitis pigmentosa, and distinctive facies) [83-91]. Additional mutations in subunits of the RNA exosome have been implicated in human disease, and diseases resulting from currently unidentified mutations in other RNA exosome subunits may be identified in future studies [92, 93].

Mutations or alterations in expression of RNA exosome cofactors, including the human orthologs of the yeast NNS complex, have been implicated in multiple human diseases. SCAF4/8 (Nrd1) has been implicated in a neurodevelopmental disorder [94], RALY (Nab3) in neuropathic pain and colorectal cancer [95, 96], and SETX (Sen1) in at

least two different neurodegenerative diseases [81, 97-99]. Additionally, mutations in the gene encoding the RNA exosome cofactor RBM7 of the human NEXT complex can cause spinal motor neuropathy [100]. Finally, mutations in genes encoding two components of the SKI cofactor complex, SKIV2L and TTC37 (Ski2 and Ski3 in *S. cerevisiae*), have been identified in a disease causing syndromic diarrhea, or trichohepatoenteric syndrome [101-105]. Greater understanding of these RNA exosome cofactors and identification of additional cofactors and cofactor functions will provide insight into how the RNA exosome and cofactors contribute to these clinically disparate disease phenotypes.

1.14 Impairment of the RNA exosome suppresses the cold-sensitive growth phenotype of *nab2-C437S* cells

Overexpression of the structural RNA exosome subunits *RRP41* and *RRP42* suppresses the *nab2-C437S* cold-sensitive growth phenotype (Mills-Lujan et al, in preparation). Often, overexpression of a gene confers increased function of the protein; however, increased function of one protein, particularly a single subunit of a multi-subunit complex, could potentially result in either enhancement or impairment of overall cellular function. Overexpression may allow a protein to increase the rate of a particular activity within a particular context. Alternatively, overexpression of a gene/protein can impair function by resulting in abnormal protein dimerization, disruption of protein complex formation and/or function, or promiscuous activity or binding of the overexpressed protein.

Previous work from our group (Mills-Lujan et al, in preparation) revealed that RNA exosome impairment rather than enhancement suppresses the cold-sensitive growth defect of *nab2-C437S* cells. Mutants that impair RNA exosome function were overexpressed in *nab2-C437S* cells and were found to suppress the growth defect. These *RRP41* and *RRP45* channel mutants [106] impair RNA exosome function by impeding access to the central channel of the RNA exosome complex. Growth suppression by these mutants was comparable to growth suppression resulting from overexpression of wildtype *RRP41* or *RRP42*. Altering the levels of these core structural subunits may disrupt formation of the RNA exosome complex by impairing the stoichiometry of the subunits within the complex. This study raised important questions about the assembly of the RNA exosome complex, which has not been studied in detail. However, the overall finding suggests that impairing the function of the RNA exosome can suppress the *nab2-C437S* mutant. This finding is consistent with a previous study that used an approach to rapidly deplete Nab2 from the nucleus and demonstrated rapid degradation of nearly all poly(A) RNA transcripts [107]. This decay was largely mediated by the RNA exosome. In such a scenario, impairing the function of the RNA exosome could help to stabilize some transcripts and protect cells from this rapid decay.

1.15 Scope and potential implications of this project

While data suggest impairment of the RNA exosome by overexpression of RNA exosome subunits *RRP41* or *RRP42* can suppress the cold-sensitive growth defect of *nab2-C437S* cells, this high copy suppressor screen also identified several RNA exosome cofactors. How a cofactor for this complex could mediate such suppression is

unclear. Furthermore, previous studies of cells that have loss or altered function of Nab2 typically show effects on a specific subset of transcripts [33, 36], raising a key question about how a polyadenosine RNA binding protein could play critical roles in regulating the processing or stability of a specific subset of polyadenylated transcripts.

This dissertation describes work focused on defining the set of RNAs most impacted by loss of Nab2 in the budding yeast model. To probe this question, I also studied the function of RNA exosome cofactors in suppression, probing what specific function of these cofactors are required to suppress the cold-sensitive growth of *nab2-C437S* cells. Additionally, the work reported here further characterizes the relationship of the RNA exosome with Nab2 by determining how loss of Nab2 function and alteration of RNA exosome function and cofactor expression affect the transcriptome (Chapter 2). In Chapter 3, I report results showing that a key non-coding RNA, the RNA component of the telomerase enzyme, TLC1, shows a significant decrease in *nab2* mutant cells. Intriguingly, this decrease in the level of *TLC1* is not accompanied by the predicted telomere shortening or cell senescence, suggesting an as yet undefined link between Nab2 and telomere maintenance. By elucidating these relationships and transcriptomic effects, we provide insight into how Nab2 functions and, ultimately, these studies may add to our understanding of how mutations in *ZC3H14* contribute to tissue-specific disease.

Figures – Chapter 1:

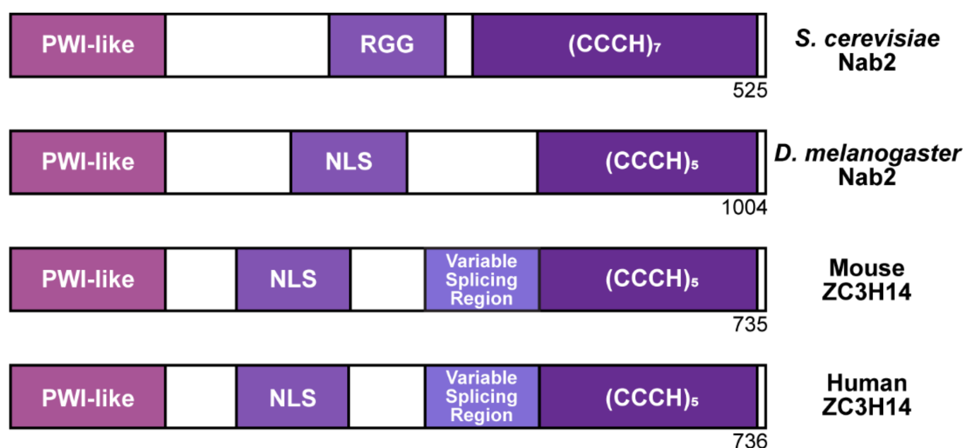

Figure 1-1: ZC3H14 domains are evolutionarily conserved. Schematic showing conserved ZC3H14/Nab2 domains. Conserved domains include an N-terminal Proline-Tryptophan-Isoleucine (PWI)-like domain that facilitates protein-protein interactions, a nuclear localization signal domain [either Arginine-Guanine-Guanine (RGG) or classical nuclear localization signal (NLS) domain], and a C-terminal CCCH zinc finger RNA binding domain containing either 5 or 7 ZnFs. The Variable Splicing Region for mouse and human ZC3H14 indicates where splicing occurs to produce four different protein isoforms. Protein lengths are indicated by the amino acid number listed under each protein domain structure.

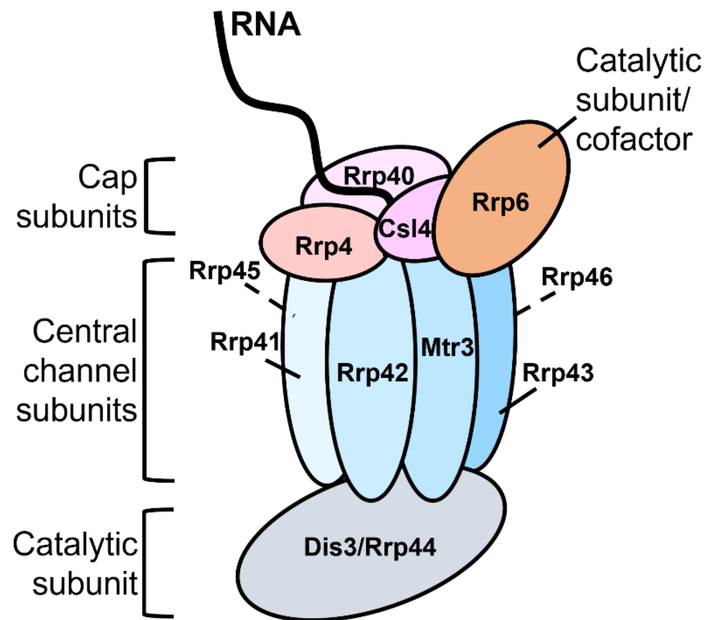

Figure 1-2: Structure of the *S. cerevisiae* nuclear RNA exosome The RNA exosome is made up of three cap subunits (Rrp4, Rrp40, Csl4), six barrel subunits (Rrp41/42/43/45/46 and Mtr3), and catalytic subunit Dis3/Rrp44. RNA exosome subunit/cofactor Rrp6 is nuclear-specific and binds to Csl4, Mtr3, and Rrp43 in a mutually exclusive manner with cytoplasmic RNA exosome cofactor Ski7 (not shown). RNA is processed or degraded by moving through the central channel to catalytic Dis3.

Chapter 2:

The RNA binding protein Nab2 genetically interacts with multiple RNA exosome cofactors to regulate target RNAs

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2.1 Abstract

RNA binding proteins play important roles in the processing and precise regulation of both coding RNAs and non-coding RNAs. Highlighting the biological importance of RNA binding proteins is the increasing number of human diseases that result from mutations in genes that encode these proteins. We recently discovered that mutations in the *ZC3H14* gene, which encodes an evolutionarily conserved polyadenosine RNA-binding protein, cause intellectual disability. Studies of the budding yeast orthologue of ZC3H14, Nuclear Poly(A) Binding protein 2 (Nab2), have provided insight into the functions of this protein. The *NAB2* gene is essential in *S. cerevisiae* and conditional *nab2* mutants cause defects in a number of steps in RNA processing. To explore the critical functions of the Nab2/ZC3H14 protein family, we performed a high-copy suppressor screen on *nab2* mutant cells. This screen identified genes encoding two core subunits of the RNA exosome, Rrp41 and Rrp42, as well as Nrd1 and Ski7, nuclear and cytoplasmic cofactors of the RNA exosome, respectively. Nrd1 is an RNA binding protein that is part of the Nrd1-Nab3-Sen1 (NNS) complex, which plays an important role in transcription termination of non-coding RNAs. Ski7 is a GTP-binding protein that mediates interaction between the RNA exosome and the Ski complex, which targets RNA transcripts to the exosome for processing and degradation in the cytoplasm. To explore the functional interactions between the RNA exosome and Nab2, we employed RNA-seq analysis to identify the coding and noncoding transcripts most impacted by overexpression of these exosome cofactors in *nab2* mutant cells. This analysis revealed that many transcripts show small changes in steady-state levels, consistent with a global role of Nab2 in modulating

transcript stability. This study uncovers functional interactions between the RNA exosome and Nab2 in both the nucleus and the cytoplasm.

2.2 Introduction

RNA binding proteins comprise a diverse set of proteins critical for many aspects of post-transcriptional control of gene expression both in the nucleus and the cytoplasm. These proteins mediate key nuclear processing events, facilitate RNA export from the nucleus, regulate translation, and perform many other equally crucial cellular functions [9]. Consistent with the numerous functions played by this class of protein, individual RNA binding proteins often contribute to multiple post-transcriptional regulatory steps required to fine tune gene expression. The importance of RNA binding proteins for proper cellular function is evident in the fact that many of these proteins are evolutionarily conserved [10] and the growing number of genes that encode RNA binding proteins that have been linked to human disease [21-23, 32]. Due to the evolutionary conservation of RNA binding proteins, a variety of model systems can be deployed to define their function and understand their multi-faceted roles in gene expression. An example of an evolutionarily conserved, multi-functional RNA binding protein linked to human disease is the zinc finger polyadenosine (polyA) RNA binding protein termed ZC3H14 in humans and Nab2 in budding yeast. Recent studies have identified mutations in *ZC3H14* that cause a non-syndromic autosomal recessive form of intellectual disability [23]. Mutations in the essential *NAB2* gene in budding yeast cause defects in mRNA splicing [36], nuclear accumulation of poly(A) RNA [43, 44], and extended poly(A) tails [24]. Rapid depletion of Nab2 from the nucleus causes a striking decrease in the level of polyadenylated RNAs [108, 109]. Nab2, which is primarily localized to the nucleus, but can shuttle between the cytoplasm and the nucleus [39] binds with high affinity to polyadenosine RNA through a zinc finger poly(A) RNA-binding domain [24, 28, 48]. How this polyadenosine RNA binding protein regulates multiple

aspects of gene expression at the mechanistic level is not yet understood.

We exploited a yeast genetics approach to identify the most critical cellular functions of the Nab2 protein. For this work, we utilized a cold-sensitive mutant of *NAB2*, *nab2-C437S* [28, 48, 49]. This amino acid substitution, which is located in the poly(A) RNA binding domain, significantly reduces binding of Nab2 to target RNAs [28, 48]. A high-copy suppressor screen was performed with *nab2-C437S* cells to identify genetic interactors of Nab2. This screen identified both core components of the RNA exosome and nuclear and cytoplasmic RNA exosome cofactors (Mills-Lujan et al., in preparation). All subunits of the RNA exosome were subsequently tested as suppressors, revealing the specificity for suppression by *RRP41* and *RRP42*. A likely mechanism for suppression is impairment of RNA exosome function by destabilization of formation of the complex upon overexpression of Rrp41 or Rrp42 [110]. Due to the conservation of these two subunits as far back as the archaeal exosome [70], the stoichiometry of the complex may rely on their precise regulation.

To test the hypothesis that impairment rather than enhancement of RNA exosome function suppresses the cold-sensitive growth phenotype, exosome mutants [106] were overexpressed in *nab2-C437S* cells (Mills-Lujan et al., in preparation). These variants contain mutations that impede procession of RNA through the central channel of the RNA exosome [106]. When overexpressed, these exosome variants suppress the *nab2-C437S* growth phenotype similarly to overexpression of *RRP41* or *RRP42,* supporting the RNA exosome impairment hypothesis. The specificity of exosome subunits in this rescue of growth indicates the importance of the RNA exosome in

mediating the growth of Nab2 mutant cells, potentially pointing to a key genetic interaction between the complex and Nab2.

While elucidating the mechanism of suppression by subunits of the RNA exosome, the previous study did not delve into the mechanism of suppression by the RNA exosome cofactors also identified in the screen. Here we focus on exploring how the overexpression of RNA exosome cofactors can suppress the cold sensitive growth of nab2 mutant cells, providing insight into both the function of the evolutionarily conserved Nab2 protein and the cofactors that regulate RNA exosome function.

2.3 Results

2.3.1 Specific RNA exosome cofactors suppress the growth defect of *nab2-C437S* cells.

A previous screen for high-copy suppressors of the *nab2-C437S* cold-sensitive phenotype (Mills-Lujan et al, in preparation) identified RNA exosome cofactors as putative high copy suppressors. The RNA exosome is a conserved ribonuclease complex with both exonuclease and endonuclease activities [53, 58, 76, 106]. It is localized to the nucleus, nucleolus, and cytoplasm, functioning in RNA processing and quality control pathways. RNA exosome cofactors assist the exosome by directing it to particular RNA targets to perform specific functions. To test whether RNA exosome cofactors can suppress *nab2-C437S* cold-sensitive growth, we performed a 10-fold serial dilution growth assay (Figure 2-1A). *nab2-C437S* cells were transformed with vector alone or, as a control, *NAB2*, as well as RNA exosome subunit genes *RRP41* and *RRP42*, which have been validated as suppressors of *nab2-C437S* (Mills-Lujan et al., in preparation). Figure 2-1A shows that the nuclear RNA exosome cofactors Nrd1, a component of the Nab3-Nrd1-Sen1 (NNS) complex which functions in transcription termination of short, primarily non-coding RNAs [111], and Ski7, which recruits the Ski complex to the RNA exosome to target aberrantly processed mRNAs for degradation and to assist in mRNA turnover [77, 112], both suppress the *nab2-C437S* growth phenotype. Nucleolar RNA exosome cofactor Nop8, which participates in processing of 5.8S pre-rRNA and, ultimately, biogenesis of the 60S ribosome [113, 114], was also identified as a suppressor.

To assess whether suppression of *nab2* growth defects is a general property of RNA exosome cofactors, we overexpressed a panel of RNA exosome cofactors in *nab2-C437S* cells (Figure 2-1B). Nuclear cofactors include the exonuclease Rrp6 and its interacting partner Rrp47, as well as Mpp6, a cofactor involved in RNA surveillance [115]. We also overexpressed genes encoding members of the nuclear TRAMP complex: Trf4, Trf5, Air1, Air2, and Mtr4. The TRAMP complex is responsible for polyadenylation of transcripts targeted for degradation by the RNA exosome [74, 116]. We also overexpressed cytoplasmic RNA exosome cofactors Dom34 and Hbs1, which comprise the Dom34-Hbs1 ribosome dissociation complex that releases stalled ribosomes from aberrantly processed mRNAs [63, 64, 117]. In addition to cofactors Nrd1, Ski7, and Nop8, we also identified Mtr4 as a robust suppressor of the cold-sensitive growth phenotype of nab2-C437S cells. This distinct subset of RNA exosome cofactors that serve as suppressors highlights the specificity of interactors.

2.3.2 Suppression of the *nab2-C437S* cold-sensitive growth phenotype is specific to the Nrd1 subunit of the NNS complex.

Nrd1 functions primarily as part of the nuclear Nab3-Nrd1-Sen1 (NNS) complex in transcription termination of short RNAs. Nrd1 and Nab3 are essential RNA binding proteins, while Sen1 is a DNA/RNA and RNA helicase. The complex co-transcriptionally interacts with RNA Polymerase II to target transcripts for transcription termination or for degradation in coordination with the TRAMP complex and the RNA exosome. Outside of the functions in the NNS complex, Nrd1 and Nab3 are also recruited to misprocessed mRNAs in a quality control pathway and can bind independently to sites within the

genome, suggesting these proteins could have independent functions [118, 119]. To determine if suppression of the *nab2-C437S* cold-sensitive growth phenotype is Nrd1-specific, we performed a growth assay and overexpressed each NNS component in *nab2-C437S* cells. Neither overexpression of *NAB3* nor *SEN1* suppressed the *nab2-C437S* growth phenotype (Figure 2-2A). The specificity of Nrd1 as an independent suppressor of the *nab2-C437S* cold-sensitive growth phenotype, suggests a possible role for Nrd1 outside of the NNS complex.

2.3.3 The RNA binding function of Nrd1 is required for *nab2-C437S* suppression.

As illustrated in Figure 2-2B, Nrd1 contains three functionally important domains: the RNA polymerase II C-terminal interacting domain (CID); the Nab3 binding domain (NBD), and the RNA recognition motif (RRM). The Nrd1 CID interacts in a mutually exclusive manner with either the C-terminal domain of Polymerase II or with Trf4 of the TRAMP complex. This domain is also responsible for direct interactions between Nrd1 and the NNS partner Sen1 as well as with RNA exosome cofactors Rrp6 and Mpp6. The NBD mediates interactions with Nab3 while the RRM mediates RNA binding to GUA[A/G] termination elements of RNAs, as well as other G-rich and AU-rich sequences [73, 111, 120, 121].

We performed a serial dilution spotting assay to determine which functional domains and interactions of Nrd1 are required for suppression of the cold-sensitive growth phenotype of *nab2-C437S* cells. Although Nrd1 is essential, both the CID and NBD domains can be deleted and the protein remains functional [73]. Thus, we generated Nrd1 deletion variants for the CID and the NBD. The Nrd1 RRM is essential

[73]. However, structural studies have defined critical residues within the RRM that mediate RNA binding (Fig 2-2C) and subsequent biochemical studies were performed to demonstrate that specific residues (F342A, H376A, and F378A) within the Nrd1 RRM are required for high affinity binding to RNA [122]. Thus, we could take advantage of this information to generate characterized Nrd1 variants that disrupt the Nrd1/Pol II interaction and the Nrd1 interaction with RNA. As shown in Figure 2D, the Nrd1 ∆CID and ∆NBD variants both suppressed the growth phenotype of *nab2-C437S* cells, while each of the Nrd1 RRM variants failed to suppress (Figure 2-2D). This result indicates that the interaction of Nrd1 with target RNAs specifically is required to suppress the *nab2* growth defect. The finding that the Nab3 binding domain of Nrd1 is dispensable for suppression further bolsters the idea that this suppression could be mediated in a Nab3-independent manner.

2.3.4 Suppression of the *nab2-C437S* cold-sensitive growth phenotype is specific to Ski7 and not subunits of the Ski complex

In the cytoplasm, the RNA exosome works in conjunction with the Ski complex to degrade non-stop RNAs [76, 123, 124]. The Ski complex is a tetramer comprised of the helicase Ski2, the tetratricopeptide protein Ski3, and two copies of the WD repeat protein Ski8 [125]. The interaction between these two complexes is mediated by Ski7. To test whether the suppression of cold-sensitive growth was specific to *SKI7* overexpression, we overexpressed the genes encoding each of the 3 different subunits of the Ski complex in *nab2-C437S* cells. While overexpression of *SKI7* showed clear

suppression, overexpression of *SKI2*, *SKI3*, and *SKI8* did not rescue *nab2-C437S* growth (Figure 2-3A).

2.3.5 The interaction between Ski7 and the RNA exosome is required for suppression of the *nab2-C437S* growth phenotype.

Ski7 has two functionally important domains, which include the N-terminal domain and the C-terminal domain (Figure 2-3B). The N-terminal domain is critical for interactions between Ski7 and both the ski complex and exosome complex [77, 112, 126, 127]. These interactions occur in distinct regions of the N-terminal domain. The C-terminal domain sequence encodes a pseudo-GTPase with a proposed role in degradation of non-stop RNAs [127]. To determine which domains of Ski7 are required for suppression of the *nab2* growth phenotype, we generated Ski7 variants consisting of either the N-terminal RNA exosome interacting domain or the C-terminal GTPase domain. We then generated variants of the N-terminal domain consisting of either the functional Ski complex-interacting domain or the functional exosome-interacting domain based on structural studies of Ski7 [128]. The Ski7 variant with a functional Ski complex-interacting domain (*ski7-exomut23*) combines amino acid changes in two of four patches in the Ski7 N-terminal exosome-interacting domain, as defined by biochemical studies [128]. Disrupting the amino acid sequences of these interaction patches has been shown to eliminate binding between Ski7 and the exosome. The *ski7 ∆ski* variant, which retains a functional exosome-interacting domain, eliminates interaction between Ski7 and the Ski complex. We performed a serial dilution spotting assay and found that the N-terminal domain of Ski7 is required for suppression of the

nab2-C437S cold-sensitive growth phenotype, indicating the requirement for interaction between Ski7 and the Ski complex, the exosome, or both (Figure 2-3C). Additionally, we found that the interaction between Ski7 and the exosome, but not the interaction between Ski7 and the Ski complex, is required for suppression of the *nab2* growth defect (Figure 2-3C).

2.3.6 Overexpression of suppressors results in altered transcript profiles.

RNA-seq analysis was performed to elucidate the transcripts and functional categories of transcripts affected in *nab2-C437S* variant cells, as well as the transcripts rescued by overexpression of suppressor genes *NRD1*, *SKI7*, *RRP41*, and *RRP42*. Principal component analysis (PCA) on normalized transcript counts displays the tight clustering of biological replicates as well as the distinctness or relatedness of the different genotypes (Figure 2-4A).

Displayed in the heatmap (Figure 2-4B), hierarchal clustering of normalized counts for combined biological replicates also shows the relatedness of genotypes. The transcript profile of *nab2-C437S* cells is altered significantly from that of wildtype cells. Overexpression of *NAB2* in the *nab2-C437S* background results in a transcript profile most similar to wildtype control cells, suggesting possible restoration of steady-state levels of transcripts altered by Nab2-C437S. In contrast, overexpression of suppressor genes *NRD1*, *SKI7*, *RRP41*, and *RRP42* in *nab2-C437S* cells results in distinct transcript profiles compared to overexpression of *NAB2*. Overexpression of suppressors *RRP41* and *RRP42* results in extremely similar transcript profiles. This is not surprising, as both suppressor genes encode subunits of the RNA exosome. Overexpression of

NRD1 results in a fairly strong degree of transcript profile overlap with the *RRP41* and *RRP42* genotypes; however, as an RNA exosome cofactor rather than subunit, *NRD1* overexpression is not as closely clustered to the exosome subunit samples as the subunit overexpression samples are to each other. Overexpression of *SKI7* results in the most distinct transcript profile, clustering away from the other suppressors and displaying the most extreme steady-state level changes of transcripts of all samples. The effects of overexpression of cytoplasmic RNA exosome cofactor *SKI7* on RNAs in the cytoplasm may account for this distinct profile.

Differential expression analysis was subsequently performed on samples (Figure 2-4C). Samples were both compared to wildtype expression levels and to *nab2-C437S* expression levels. Like the normalized counts, the correlations between samples show the highest degree of relatedness between the *RRP41* and *RRP42* overexpression samples. The *NRD1* overexpression sample closely correlates with the subunits, while *SKI7* is only moderately correlated to any other sample.

2.3.7 The *nab2-C437S* mutation affects a small subset of gene ontology categories.

Nab2-C437S cells have sets of transcripts with both increased and decreased steady-state levels (Figure 2-5A). The majority of affected transcripts show a $\log_2$ fold change smaller than 1. To determine the gene ontology categories affected by nab2-C437S, Gene Set Enrichment Analysis (GSEA) was performed. This analysis revealed 14 gene ontology (GO) categories significantly decreased in *nab2-C437S* cells compared to wildtype (Figure 2-5B). Of these GO terms, several overarching categories

encapsulate multiple listed terms. These include ribosome subunits and biogenesis, polymerase activity, and telomere maintenance. Zero GO terms were significantly increased compared to wildtype cells. Overexpression of *NAB2* resulted in rescue of most significantly decreased GO categories in *nab2-C437S* cells (Figure 2-5C), highlighting the importance of Nab2 function in regulating these cellular processes. However, overexpression of suppressor genes *NRD1*, *SKI7*, and *RRP41* did not show rescue of these categories (Figure 2-6), suggesting an alternative mode of suppression.

2.3.8 Overexpression of suppressors results in significant overlap of affected transcripts and GO categories

Overexpression of suppressors *NRD1*, *SKI7*, and *RRP41* in *nab2-C437S* cells resulted in overlapping sets of affected transcripts among suppressors (Figure 2-7). Transcripts with significantly increased or decreased steady-state levels resulting from *RRP41* overexpression are largely and similarly altered by *NRD1* overexpression, showing an overlap of 89%. While the majority of transcripts affected by *RRP41* overexpression were also affected by *NRD1* overexpression, *NRD1* overexpression impacted a large number of transcripts unaffected by RRP41 overexpression. Transcripts affected by *NRD1* overexpression share a greater overlap with transcripts affected by *SKI7* overexpression than with transcripts affected by *RRP41* overexpression. The overlap of transcripts altered by overexpression of suppressor gene *RRP41*, which is both nuclear and cytoplasmic, with transcripts affected by overexpression of nuclear RNA exosome cofactor *NRD1* suggests that the most critical transcripts for suppression may be nuclear.

Following a similar trend of overlap as transcripts, Gene Set Enrichment Analysis showed overlap of GO categories among suppressor samples (Figure 2-8A). To determine the similarities in categories most affected by *NRD1* or *SKI7* overexpression, the top ten most positively and negatively enriched GO categories were compared among samples (Figure 2-8B-C). Of the categories most decreased by *NRD1* overexpression, all were similarly decreased by overexpression of *SKI7* or *RRP41*. Of the categories most increased by *NRD1* overexpression, all were similarly increased by overexpression of *RRP41*. Half of these increased categories were also increased upon *SKI7* overexpression; however, categories pertaining to the cytosol were decreased. Of the categories most decreased by *SKI7* overexpression, all were similarly decreased by *NRD1* overexpression. Notably, overexpression of *RRP41* resulted in an increase for nine out of ten of these categories. Of the categories most increased by SKI7 overexpression, all categories were similarly increased by either *NRD1* or *RRP41* overexpression.

2.3.9 Transcript levels altered by overexpression of suppressors are not altered in wildtype control cells.

Overexpression of *NRD1*, *SKI7*, or *RRP41* alters the steady-state levels of thousands of genes in *nab2-C437S cells*. As elucidating the functions and interactions of Nab2 was the main goal of this project, a concern arose that, rather than shedding light on Nab2 function, the RNA-seq data was revealing how RNA exosome impairment independently affects the transcriptome. To determine the extent to which overexpression of cofactors and subunits affects the transcriptome independent of

Nab2, we performed RNA-sequencing on samples overexpressing *NRD1*, *SKI7*, or *RRP41* in wildtype control cells. Surprisingly, we found significantly fewer affected transcripts in control cells and little overlap of altered transcripts between samples analyzed (Figure 2-9). *NRD1* overexpression in the control cells significantly alters the steady-state levels of 13 transcripts, compared to almost 4000 affected transcripts in *nab2-C437S* cells. *SKI7* overexpression increases the steady-state levels of 149 transcripts, while only 5 have decreased steady-state levels in control cells. Finally, *RRP41* overexpression affects the steady-state levels of 273 transcripts in the control cells, the greatest number amongst the suppressors, but this number is less than ten percent of the total number of transcripts increased or decreased in the *nab2-C437S* cells. Of the 273 transcripts, only 63 are affected in both backgrounds.

2.3.10 Overexpression of *NRD1* and *SKI7* may suppress through partially different mechanisms

Although overexpression of *NRD1* or *SKI7* likely results in RNA exosome impairment, the method or amount of impairment may differ between cofactors. To address this question, we utilized a genetic approach by co-overexpressing *NRD1* and *SKI7* in the same cells and comparing growth to overexpression of *NRD1* or *SKI7* alone (Figure 2-10). If both cofactors suppress through the same mechanism, we would expect similar growth upon co-overexpression in the *nab2-C437S* cells. Strikingly, co-overexpression results in improved growth of the *nab2-C437S* cells compared to either cofactor overexpressed alone.

We also co-overexpressed either *NRD1* or *SKI7* together with *RRP41* (Figure 2-10). We found that co-overexpression with *RRP41* improved growth as compared to *nab2-C437S* cells with *NRD1* or *SKI7* overexpressed alone. However, co-overexpression did not further enhance growth of the nab2-C437S cells compared to *RRP41* overexpression alone.

2.4 Discussion

In a previously described screen (Mills-Lujan et al., in preparation), we identified specific RNA exosome subunits and cofactors that suppress the cold-sensitive growth phenotype seen in *nab2-C437S* mutant cells. Rrp41 and Rrp42, two structural components of the core RNA exosome, were identified as suppressors. Analysis of the mechanism of suppression suggests that overexpression of these individual subunits of the complex suppress by decreasing RNA exosome activity, potentially due to destabilizing complex formation by disrupting subunit stoichiometry. Further supporting the hypothesis of suppression by RNA exosome impairment is the discovery of suppression of *nab2-C437S* cold-sensitive growth by structural RNA exosome mutants that impede transcript progression through the central channel of the complex.

Impairment of the RNA exosome can interrupt numerous functions of the complex and impede processing of various classes of RNAs. In the nucleus and nucleolus, the RNA exosome largely serves to process non-coding RNAs, including pre-rRNAs, tRNAs, and snoRNAs. It also functions in turnover of RNAs and degradation of misprocessed pre-mRNAs in the nucleus [54, 55, 106]. In the cytoplasm, the RNA exosome functions in mRNA turnover and in the quality control pathway responsible for degrading aberrant mRNAs resulting from non-stop decay, nonsense-mediated decay, and no-go decay [58, 61, 129].

The diversity of roles performed by the RNA exosome among such a wide array of potential RNA targets requires careful coordination of recruitment and activities. This coordination is facilitated by cofactors. Just as there are diverse roles performed by the RNA exosome, cofactors modulate exosome function through a variety of mechanisms.

The specificity of exosome cofactors that also suppress the cold-sensitive growth phenotype of *nab2-C437S* cells upon overexpression may also distinguish particular functions of the exosome that are critical for Nab2 function, as well as Nab2 target RNAs that are crucial for growth.

The isolation of Nrd1, Ski7, Nop8, and Mtr4 as the RNA exosome cofactors able to suppress the *nab2-C437S* growth defect upon overexpression implicates their interactions with the RNA exosome as particularly important for the interplay of the complex with Nab2. Intriguingly, these cofactors are nuclear, cytoplasmic, nucleolar, and nuclear, respectively, and they function in distinct cellular contexts. Nrd1, Ski7, and Nop8 were identified in the original high-copy suppressor screen, while Mtr4 was identified in a later experiment. For the purposes of this study, Nrd1 and Ski7 were focused on due both to their identification in the initial screen and to the plethora of foundational research already published on their structures and functions.

Although Nop8 was not the focus of this study, its identification as a suppressor does provide supporting evidence for the hypothesis that impairment rather than enhancement of RNA exosome function is responsible for suppression. Nop8 is an essential nucleolar RNA exosome cofactor that negatively regulates activity of the RNA exosome by binding Rrp6 in a concentration-dependent manner. Nop8 functions in opposition to RNA exosome activator Nop53 in the processing of 5.8S pre-rRNA and, ultimately, biogenesis of the 60S ribosome. Nop8 contains an N-terminal domain where it interacts with 5.8S rRNA and a coiled C-terminal domain where it interacts with Nip7, a nucleolar protein which physically interacts with core exosome subunit Rrp43, and Rrp6 [114]. As RNA exosome activity is negatively regulated by increasing expression

of Nop8, the overexpression of *NOP8* in *nab2-C437S* cells is likely also down-regulating exosome activity.

To further investigate the effect of *nab2* mutation and subsequent overexpression of suppressors, we performed RNA sequencing analysis. We identified fourteen gene ontology categories negatively impacted by the *nab2* mutation. While these categories are largely rescued by *NAB2* overexpression, they are not rescued by overexpression of the suppressors. This suggests the suppressors rescue growth by other mechanisms. Gene sets affected by overexpression of *NRD1*, *SKI7*, and *RRP41* show a large percentage of overlap. This overlap may indicate a common mechanism of suppression. However, when *NRD1* and *SKI7* are co-overexpressed in *nab2-C437S cells*, rescue of growth is improved compared to overexpression of either cofactor individually. This finding suggests that suppression may be achieved through at least partially different mechanisms.

When either *NRD1* or *SKI7* are co-overexpressed with *RRP41* in *nab2-C437S* cells, growth is improved compared to overexpression of either cofactor alone but not overexpression of *RRP41* alone. This finding suggests that impairment of the RNA exosome is greater when a core subunit of the RNA exosome is overexpressed than when a cofactor is overexpressed. As subunits are more central to RNA exosome function than auxiliary cofactors, it makes sense that overexpression of *RRP41* would alter the stoichiometry of the RNA exosome complex, impair the function of the RNA exosome, and thus confer suppression of cells where Nab2 function is sub-optimal, such as the *nab2-C437S* cells.

While studying the impact of RNA exosome cofactor and subunit overexpression, a concern arose that our findings could be elucidating how the RNA exosome affects transcripts regardless of Nab2. By performing RNA-seq analysis on control cells overexpressed with *NRD1*, *SKI7*, or *RRP41*, we found that overexpression of suppressors outside of the impaired Nab2 context has little effect on the transcriptome when compared to control cells without suppressors. Furthermore, we found very little overlap between transcripts affected by suppressors in *nab2-C437S* cells versus control cells. The minimal amount of overlap between the *nab2-C437S* and control cells overexpressing each of these suppressors suggests an important interplay between Nab2-C437S and the RNA exosome. To whatever extent the RNA exosome is impaired by overexpression of cofactors or subunits, this impairment has little impact on the steady-state levels of target RNAs when Nab2 function is normal, as in the control cells employed. This finding provides evidence that the transcriptomic data acquired is relevant to elucidating Nab2 function and characterizing the interactions of Nab2 with Nrd1, Ski7, and Rrp41.

The RNA exosome is localized to both the nucleus and the cytoplasm, but its function is required in the nucleus where it serves as the primary degradation pathway. Conversely, in the cytoplasm, the main degradation pathway utilizes the 5’ to 3’ exonuclease Xrn1 [130]. While the RNA exosome functions in both compartments, Nrd1 and Ski7 localize to the nucleus and cytoplasm, respectively. Nuclear-localized Nrd1 assists the RNA exosome in processing nuclear transcripts, and overexpression of *NRD1* likely affects nuclear transcripts. Intriguingly, most transcripts affected by

overexpression of *RRP41* are also affected by *NRD1* overexpression, suggesting a primarily nuclear mechanism for suppression by *RRP41*.

Localized to the cytoplasm, Ski7 affects RNAs after they are transported from the nucleus. This may explain the additional transcripts identified as increased or decreased by *SKI7* overexpression. Overexpression of cytoplasmic *SKI7* may also affect nuclear transcripts by impacting the formation or import of the RNA exosome into the nucleus. Through its N-terminal region, Ski7 binds to the same subunits of the RNA exosome as nuclear subunit/cofactor Rrp6 binds through its C-terminal region. These exosome cofactors include Csl4, Mtr3, and Rrp43. Ski7 and Rrp6 bind the exosome in a mutually exclusive manner, and in vitro work has shown that Ski7 can outcompete Rrp6 for exosome binding [128, 131]. Overexpression of *SKI7* could impact RNA exosome function by outcompeting Rrp6 and sequestering either binding partner subunits of the exosome or the entire complex in the cytoplasm. This could result in reduced import of the RNA exosome into the nucleus or reduced nuclear localization of binding partner subunits (Figure 2-11). Either possibility could result in reduced nuclear function of the RNA exosome. Additionally, cytoplasmic sequestration of the complex could lead to enhanced function of the RNA exosome in the cytoplasm by increasing the number of complexes in that compartment. Following up on these hypotheses and elucidating the molecular mechanisms of suppression will be the focus of future studies.

An intriguing possibility is that connections of RNA exosome cofactors Nrd1, Ski7, Nop8, and Mtr4 with exosome cofactor/subunit Rrp6 may provide a common underlying pathway to suppression. Each of these cofactors interacts with Rrp6 either directly or competitively. Nrd1 and Rrp6 work together in the NNS transcription

termination pathway [132], as well as a quality control pathway for targeting misprocessed mRNAs for degradation [119]. Additionally, Nrd1 may protect transcripts from degradation by Rrp6 by preventing Rrp6 procession along Nrd1-bound RNAs (Figure 2-11) [73]. Ski7 and Rrp6 bind to the core RNA exosome in a mutually exclusive manner, potentially affecting localization of the complex [128]. Nop8 interacts directly with Rrp6 to down-regulate RNA exosome activity in the nucleolus [114]. Mtr4 is recruited to the exosome by Rrp47 and directly interacts with Rrp47 and binding partner Rrp6 at the exosome [133]. Mtr4 adenylation activity also competes with Rrp6 deadenylation activity to determine how, depending on transcript stability, RNAs are processed or degraded [116]. These connections to Rrp6 could impact Rrp6 function, core exosome function, or both.

Figures – Chapter 2:

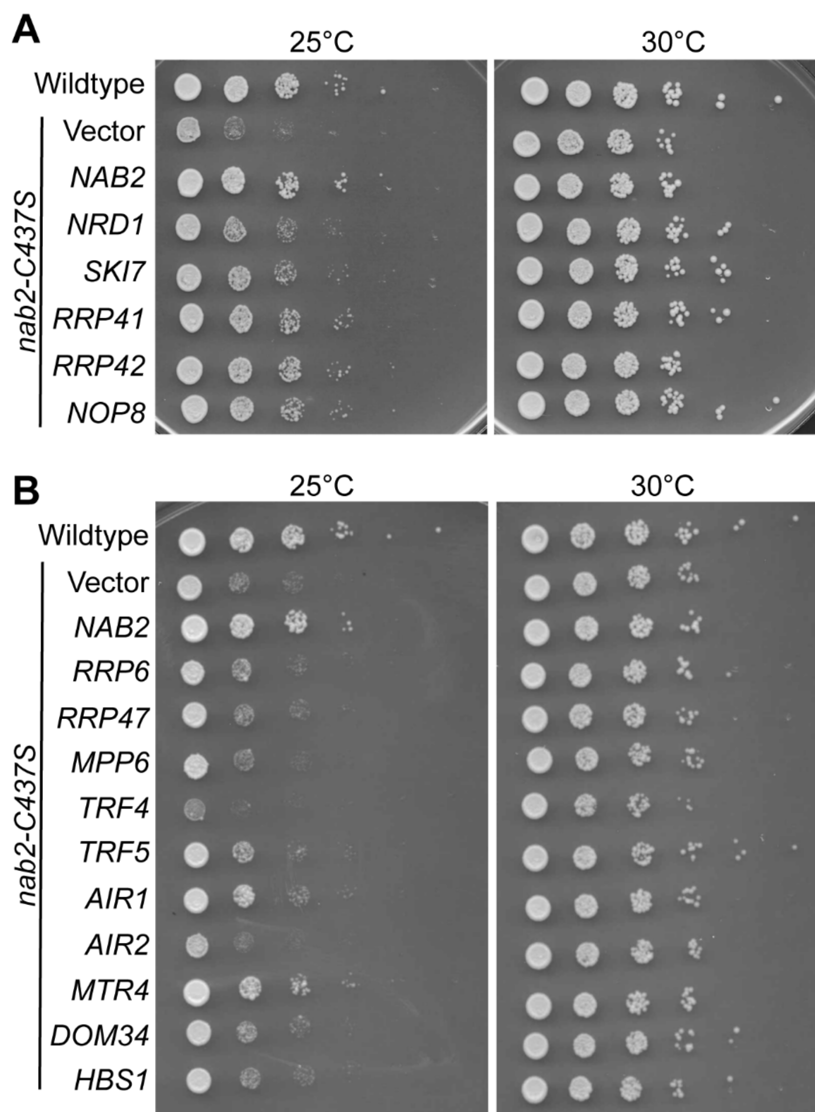


Figure 2-1: Suppression of the *nab2-C437S* cold-sensitive growth defect is specific to RNA exosome cofactors *SKI7*, *NRD1*, *NOP8*, and *MTR4*. *Nab2-C437S* cells show a cold-sensitive growth defect at 25°C. Suppressor genes were identified through a high-copy suppressor screen. (A) *nab2-C437S* cells were transformed with 2μ high-copy plasmids containing empty vector or genes identified in the high-copy suppressor screen. Cells were then serially diluted, spotted onto solid media plates, and grown at either 25°C or 30°C for 1-2 days. Overexpression of RNA exosome cofactor genes *NRD1*, *SKI7*, and *NOP8*, as well as RNA exosome subunit genes *RRP41* and *RRP42* suppress the cold-sensitive growth defect. (B) To test for specificity of RNA exosome cofactors as suppressors of the *nab2-C437S* growth defect, cells were transformed with 2μ plasmids containing nuclear and cytoplasmic cofactor genes. Cells were then serially diluted spotted onto solid media plates, and grown at either 25°C or 30°C for 1-2 days.

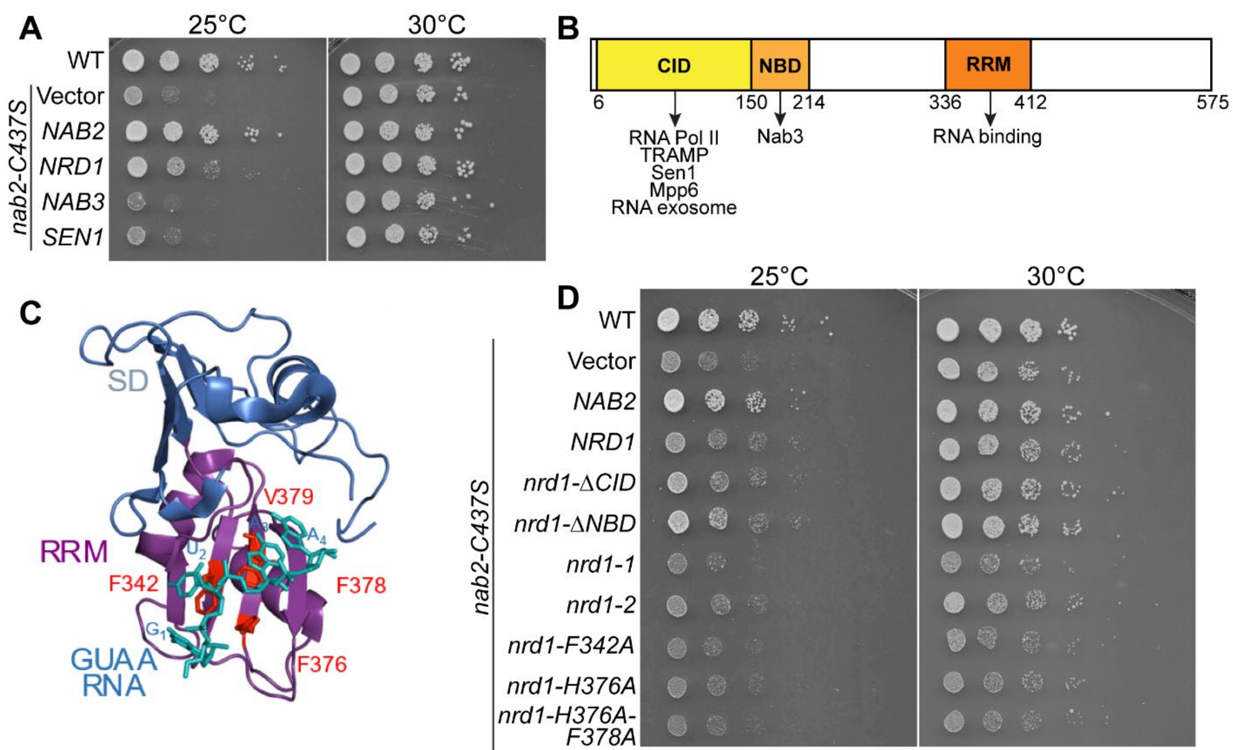

A
25°C
30°C
WT
nab2-C437S
Vector
NAB2
NRD1
NAB3
SEN1
B
CID
NBD
RRM
6
150
214
336
412
575
RNA Pol II
TRAMP
Sen1
Mpp6
RNA exosome
Nab3
RNA binding
C
SD
RRM
V379
F342
F378
F376
GUAA
RNA
D
25°C
30°C
WT
nab2-C437S
Vector
NAB2
NRD1
nrd1-ΔCID
nrd1-ΔNBD
nrd1-1
nrd1-2
nrd1-F342A
nrd1-H376A
nrd1-H376A-
F378A

Figure 2-2: Suppression is specific to *NRD1* of the Nrd1-Nab3-Sen1 (NNS) complex and requires the Nrd1 RNA binding domain. (A) To test for specificity of suppression by *NRD1*, 2µ plasmids containing NNS subunit genes *NRD1*, *NAB3*, and *SEN1* were transformed into *nab2-C437S* cells. Cells were serially diluted, spotted onto solid media plates, and grown at 25°C or 30°C for 1-2 days. (B) Nrd1 domain structure with domain-specific interactions indicated by arrows. CID, Polymerase II C-Terminal Interacting Domain; NBD, Nab3 Binding Domain; RRM, RNA Recognition Motif. (C) Crystal structure of the essential Nrd1 RRM and interacting GUAA RNA [120]. Indicated amino acids are required for RNA binding, and Nrd1 variants with impaired RNA binding function were generated by changing these amino acids. (D) To test for Nrd1 domains that are required for suppression, *nab2-C437S* cells were transformed with 2µ plasmids containing *NRD1* mutants lacking the CID (∆*CID*), lacking the NBD (∆*NBD*), or with impaired RNA binding (premature nonsense mutants *nrd1-1*, *nrd1-2*; *nrd1-F342A*, *nrd1-H376A*, *nrd1-H376A-F378A*).

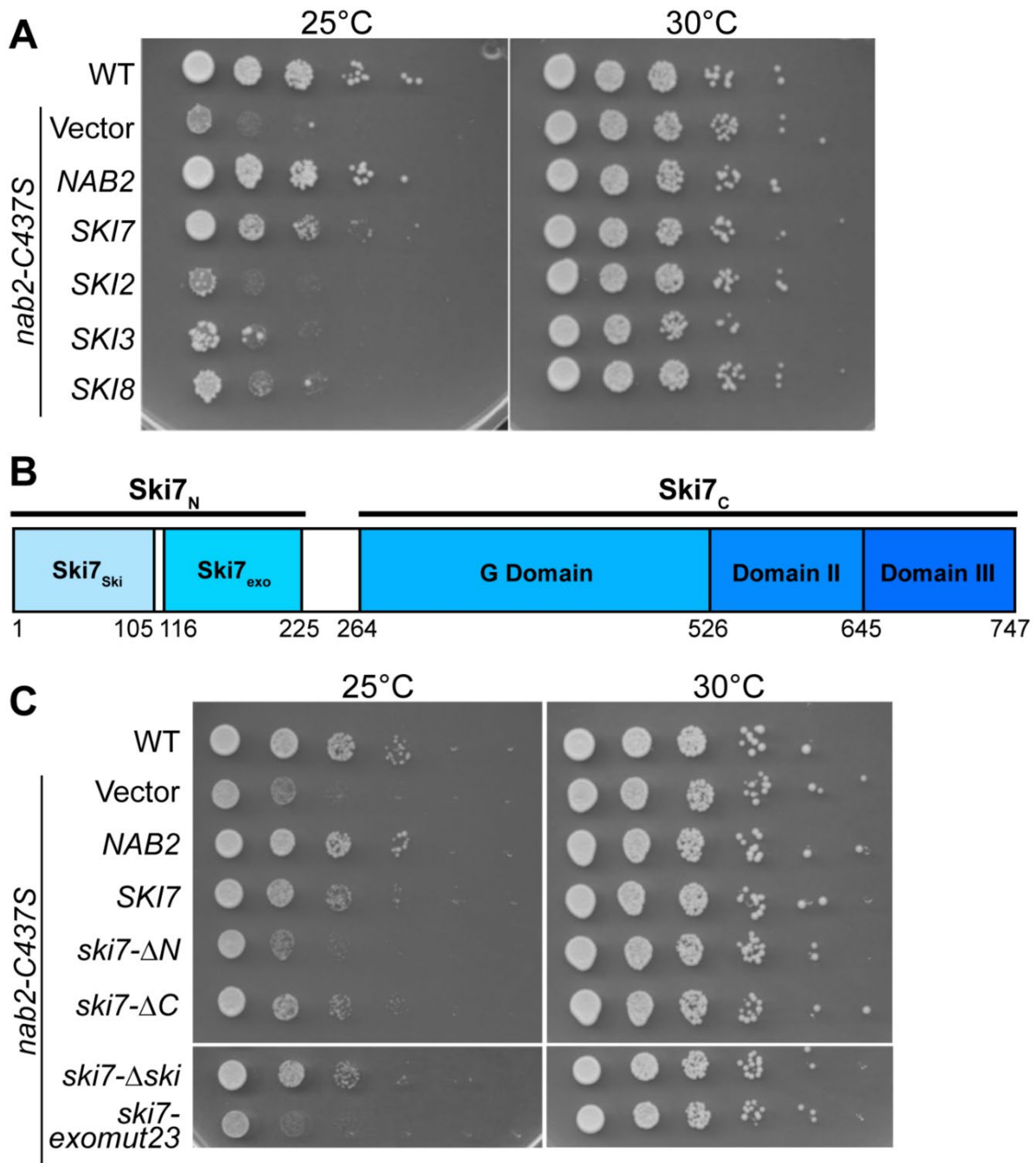

A
25°C
30°C
WT
Vector
NAB2
SKI7
SKI2
SKI3
SKI8
nab2-C437S
B
Ski7N
Ski7C
Ski7Ski
Ski7exo
G Domain
Domain II
Domain III
1
105
116
225
264
526
645
747
C
25°C
30°C
WT
Vector
NAB2
SKI7
ski7-ΔN
ski7-ΔC
ski7-Δski
ski7-exomut23
nab2-C437S

Figure 2-3: Suppression is specific to *SKI7* and requires the Ski7-RNA exosome interacting domain. (A) *nab2-C437S* cells were transformed with 2μ plasmids containing subunits of the cytoplasmic RNA exosome cofactor Ski complex. Cells were serially diluted, spotted onto solid media plates, and grown at 25°C or 30°C for 1-2 days. (B) Ski7 domain structure. $Ski7_{ski}$, Ski complex-interacting domain; $Ski7_{exo}$, RNA exosome-interacting domain. (C) *nab2-C437S* cells were transformed with 2μ plasmids containing SKI7 mutants lacking the C-terminal domain (Δ*C*), lacking the N-terminal domain (Δ*N*), lacking the N-terminal Ski-complex interacting domain (Δ*ski*), or mutations in the N-terminal RNA exosome interacting domain that interrupt binding to the RNA exosome (*exomut23*).

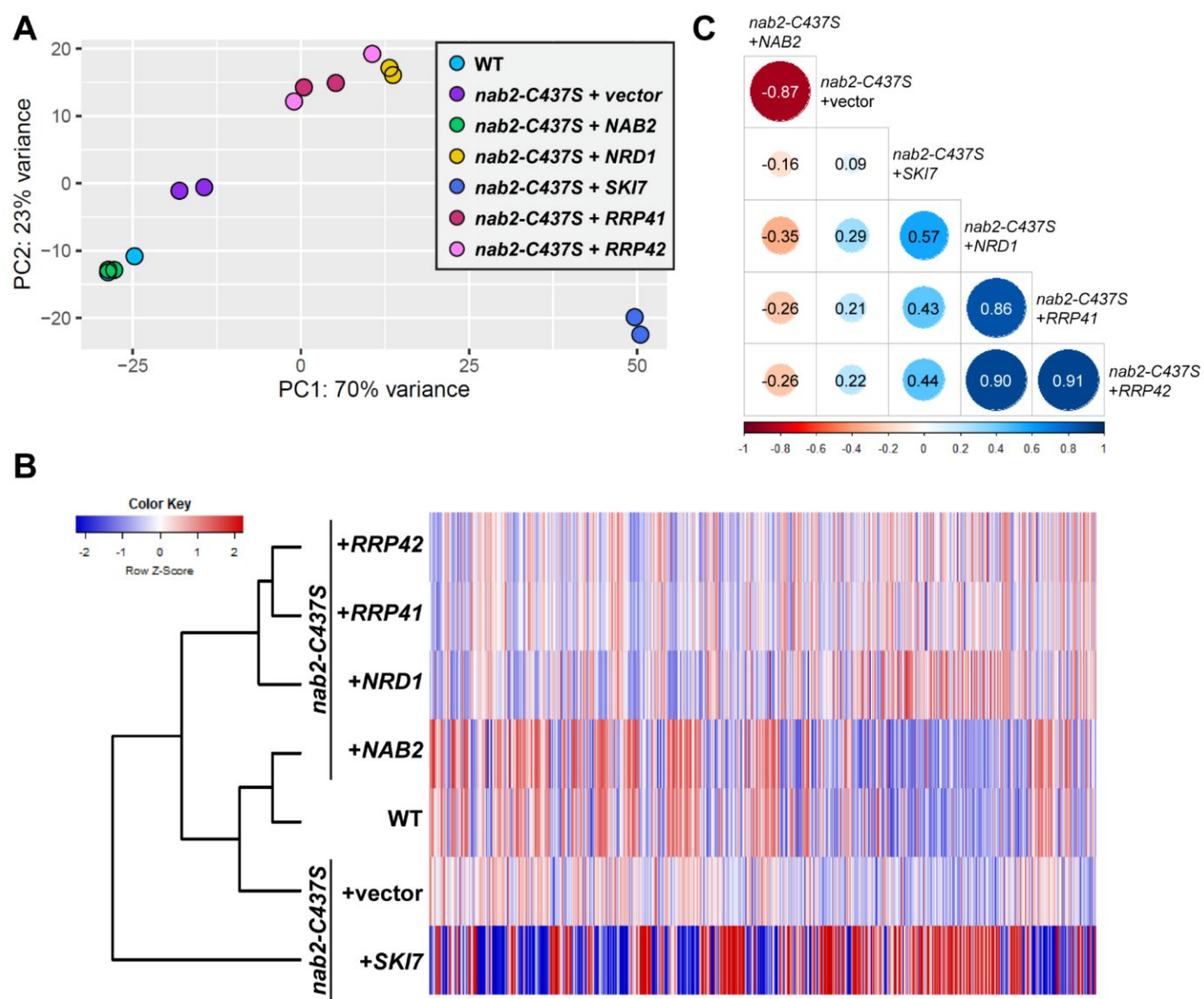

A
PC2: 23% variance
PC1: 70% variance
WT
nab2-C437S + vector
nab2-C437S + NAB2
nab2-C437S + NRD1
nab2-C437S + SKI7
nab2-C437S + RRP41
nab2-C437S + RRP42
B
Color Key
Row Z-Score
+RRP42
+RRP41
+NRD1
+NAB2
WT
+vector
+SKI7
nab2-C437S
C
nab2-C437S +NAB2
nab2-C437S +vector
nab2-C437S +SKI7
nab2-C437S +NRD1
nab2-C437S +RRP41
nab2-C437S +RRP42
-0.87
-0.16
0.09
-0.35
0.29
0.57
-0.26
0.21
0.43
0.86
-0.26
0.22
0.44
0.90
0.91

Figure 2-4: RNA-sequencing reveals distinct transcriptomes for *nab2-C437S* cells overexpressed with suppressor genes. RNA-sequencing was performed on biological duplicate samples of each genotype. (A) Principal component analysis (PCA) performed comparing rlog normalized gene counts for each biological replicate of all genotypes. (B) Heatmap showing rlog normalized gene count comparisons for combined biological replicates of each genotype. Heatmap is oriented horizontally. Each gene count is individually scaled across samples. (C) Correlation matrix showing correlations between differentially expressed genes of each genotype (combined replicates). Each genotype label corresponds to a row and column. Circle sizes correspond to degree of correlation. All correlation values are statistically significant, with statistical significance set at $p<0.05$.

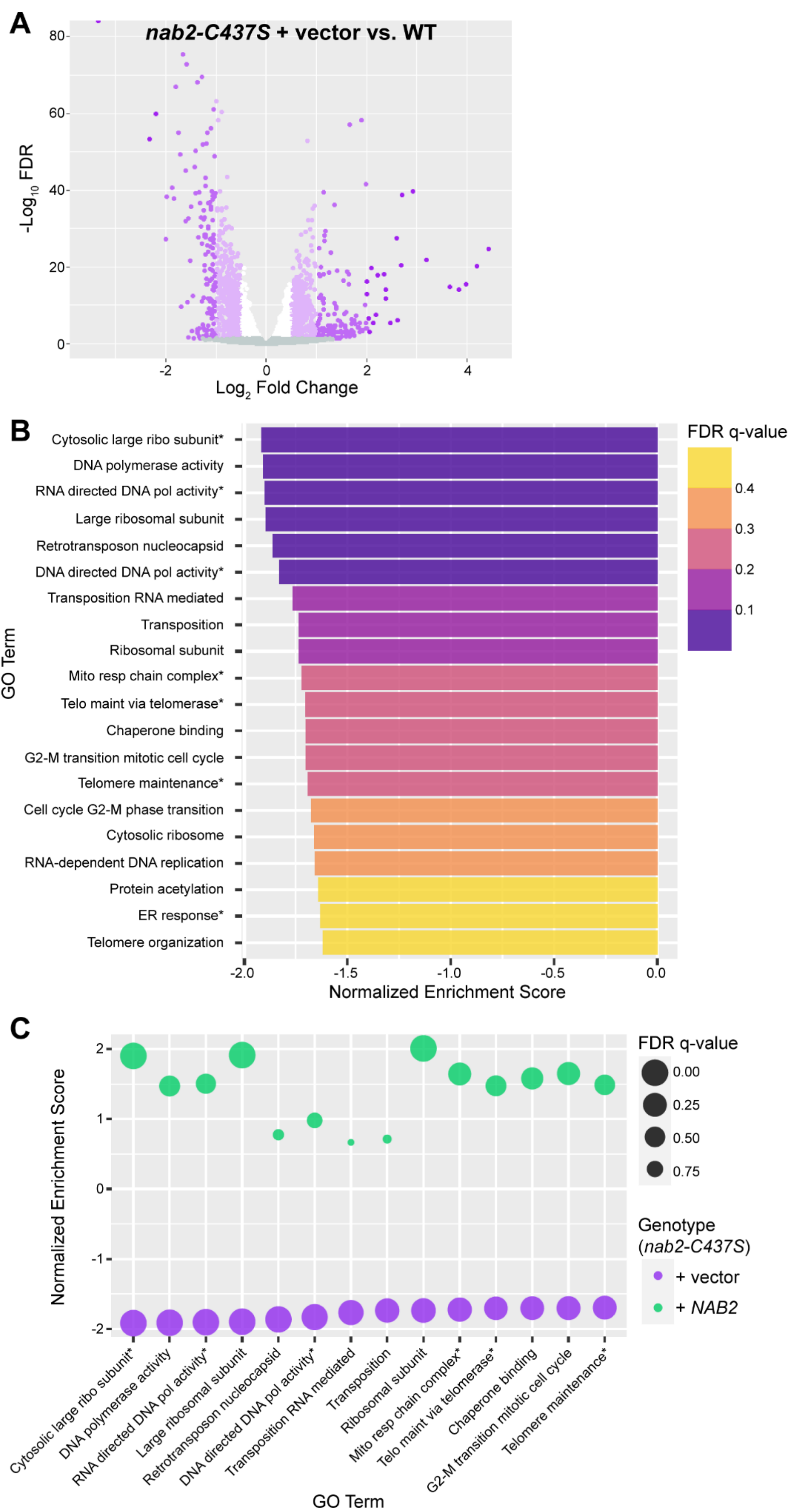

A
nab2-C437S + vector vs. WT
-Log10 FDR
Log2 Fold Change
B
Cytosolic large ribo subunit*
DNA polymerase activity
RNA directed DNA pol activity*
Large ribosomal subunit
Retrotransposon nucleocapsid
DNA directed DNA pol activity*
Transposition RNA mediated
Transposition
Ribosomal subunit
Mito resp chain complex*
Telo maint via telomerase*
Chaperone binding
G2-M transition mitotic cell cycle
Telomere maintenance*
Cell cycle G2-M phase transition
Cytosolic ribosome
RNA-dependent DNA replication
Protein acetylation
ER response*
Telomere organization
GO Term
Normalized Enrichment Score
FDR q-value
C
Normalized Enrichment Score
FDR q-value
Genotype
(nab2-C437S)
+ vector
+ NAB2
GO Term

Figure 2-5: *Nab2-C437S* cells show 14 gene ontology categories significantly down compared to control cells. (A) Volcano plot showing differentially expressed genes in *nab2-C437S* cells compared to control cells. (B) Bar chart showing the most decreased GO terms in *nab2-C437S* cells compared to control cells. Categories with an FDR q-value <0.25 (including dark purple, medium purple, and pink bars) were considered for further analyses. (C) Bubble plot showing comparison of the 14 GO categories most significantly down in *nab2-C437S* cells (compared to control cells) against *nab2-C437S* cells overexpressed with wildtype *NAB2* (compared to *nab2-C437S* cells). (B-C) Asterisk (*) used to indicate truncated category names and/or abbreviated terms. [ribo, ribosomal; pol, polymerse; telo, telomere; maint, maintenance] [Mito resp chain complex, Mitochondrial respiratory chain complex assembly; Telomere maintenance via telomere lengthening; ER response, Endoplasmic reticulum unfolded protein response & response to endoplasmic reticulum stress & cellular response to unfolded protein]

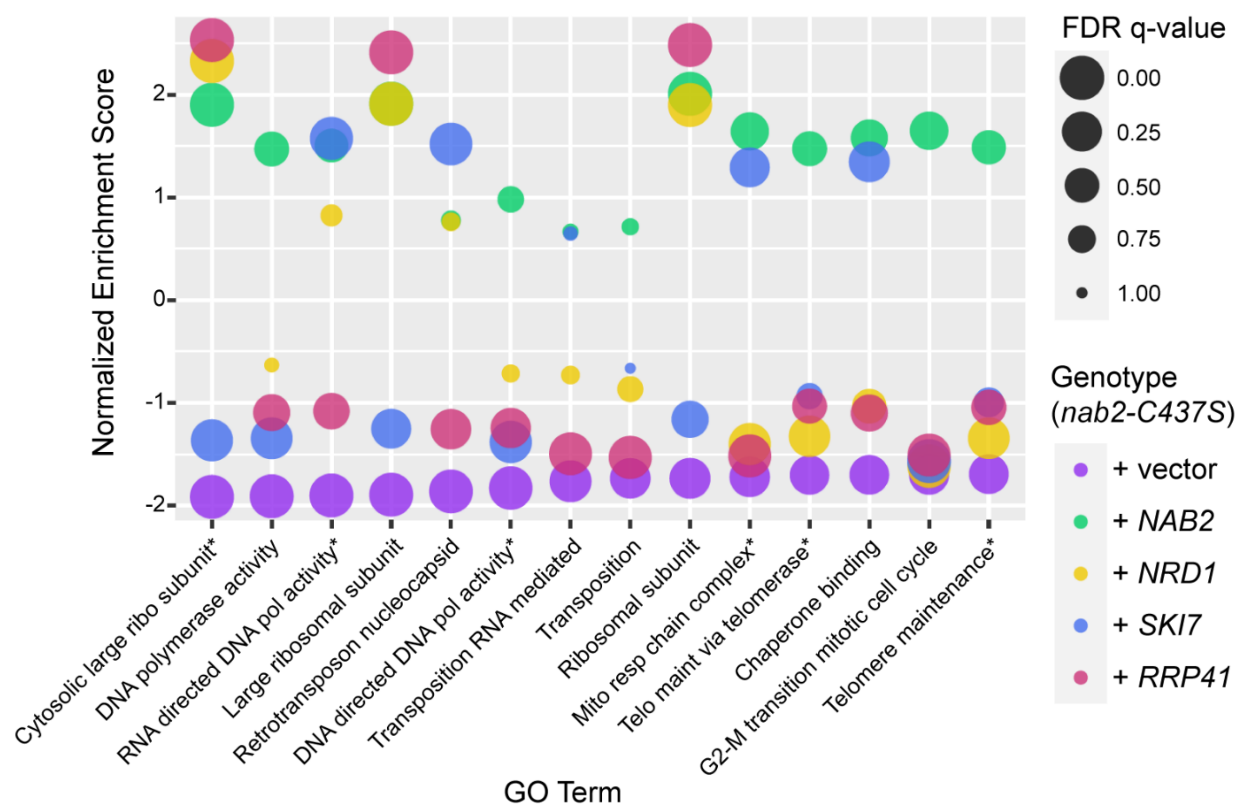


Figure 2-6: The top 14 down categories in *nab2-C437S* cells show disparate patterns of rescue/lack of rescue upon overexpression of suppressors *NRD1*, *SKI7*, and *RRP41*. Bubble plot showing comparison among samples for the 14 most significantly down categories identified in *nab2-C437S* cells. Asterisk (*) used to indicate truncated category names and/or abbreviated terms. [ribo, ribosomal; pol, polymerse; telo, telomere; maint, maintenance] [Mito resp chain complex, Mitochondrial respiratory chain complex assembly; Telomere maintenance via telomere lengthening]

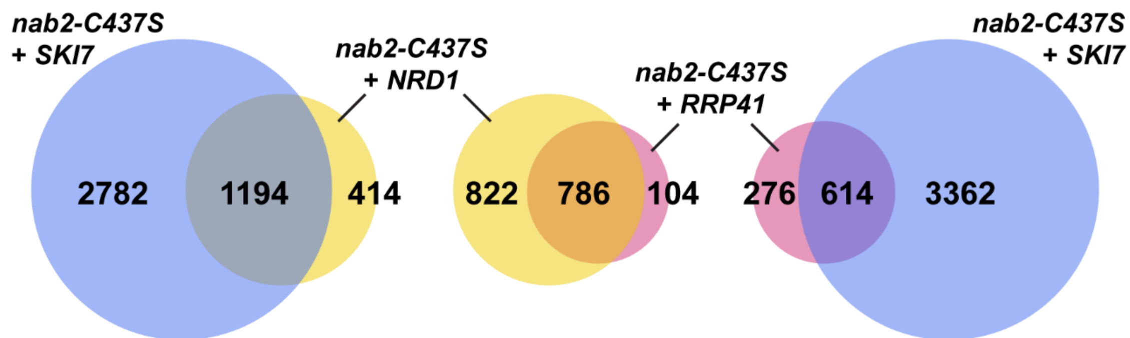
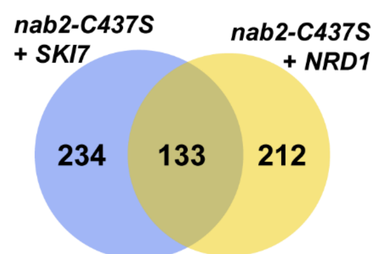
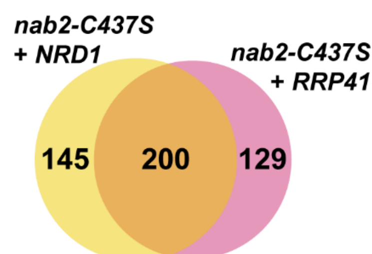


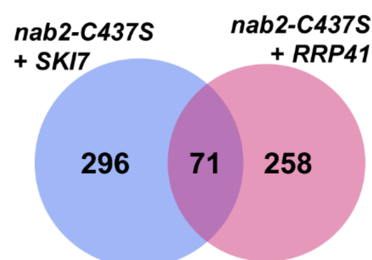
Figure 2-7: Overexpression of suppressors in *nab2-C437S* cells results in significant overlap of differentially expressed genes between different suppressor samples. Lists of differentially expressed genes for *nab2-C437S* cells overexpressed with *NRD1*, *SKI7*, or *RRP41* were compared for each pair of suppressors. Gene counts include transcripts with both up and down steady-state levels. Absolute $\log_2$ fold change cut-off set at $\alpha > 0.5$. Statistical significance set at $p < 0.05$. Circles drawn to scale.

A GO categories

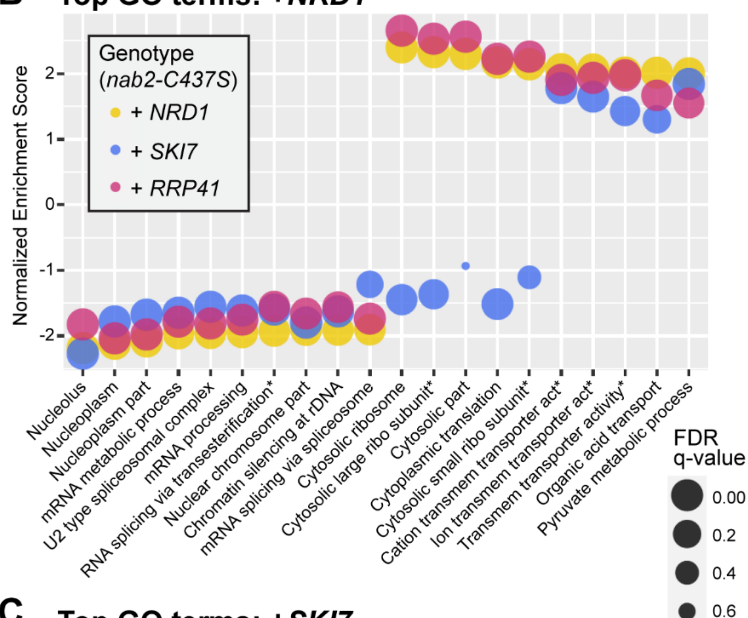
Increased + Decreased







B Top GO terms: +*NRD1*



C Top GO terms: +*SKI7*

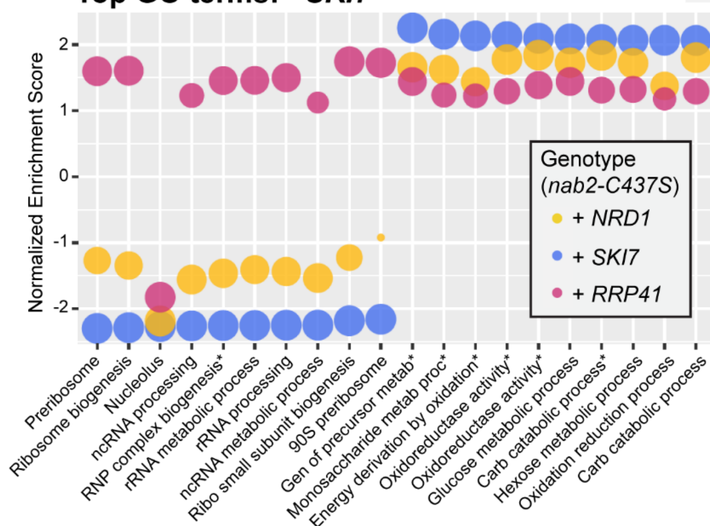


Figure 2-8: Overexpression of *NRD1*, *SKI7*, and *RRP41* results in significant overlap of differentially expressed gene ontology categories affected by each suppressor. (A) Venn diagrams showing comparisons of differentially expressed gene ontology categories comparing each pair of suppressors in *nab2-C437S* cells. Totals include both up and down gene ontology terms. Absolute log_2 fold change cut-off set at $\alpha>0.5$. Statistical significance set at $p<0.05$. Circles drawn to scale. (B) Bubble plot showing the top 10 down and top 10 up GO terms for *nab2-C437S* cells overexpressed with *NRD1*. Matching GO terms were then subset from *SKI7* and *RRP41* overexpression datasets and plotted. (C) Bubble plot showing the top 10 down and top 10 up GO terms for *nab2-C437S* cells overexpressed with *SKI7* with matching GO terms subset and plotted from *NRD1* and *RRP41* overexpression datasets. (B-C) Log_2 fold change cut-offs set at $\alpha>0.5$ or $\alpha<-0.5$. Statistical significance set at $p<0.05$. [ribo, ribosomal; transmem, transmembrane; act, activity; RNP, ribonucleoprotein; carb, carbohydrate] [Gen of precursor metab, Generation of precursor metabolites and energy; Monosaccharide metab proc, Monosaccharide metabolic process; Energy derivation by oxidation of organic compounds; Oxidoreductase activity acting on CH OH group of donors; Oxidoreductase activity acting on the CH OH group of donors NAD or NADP as acceptor]

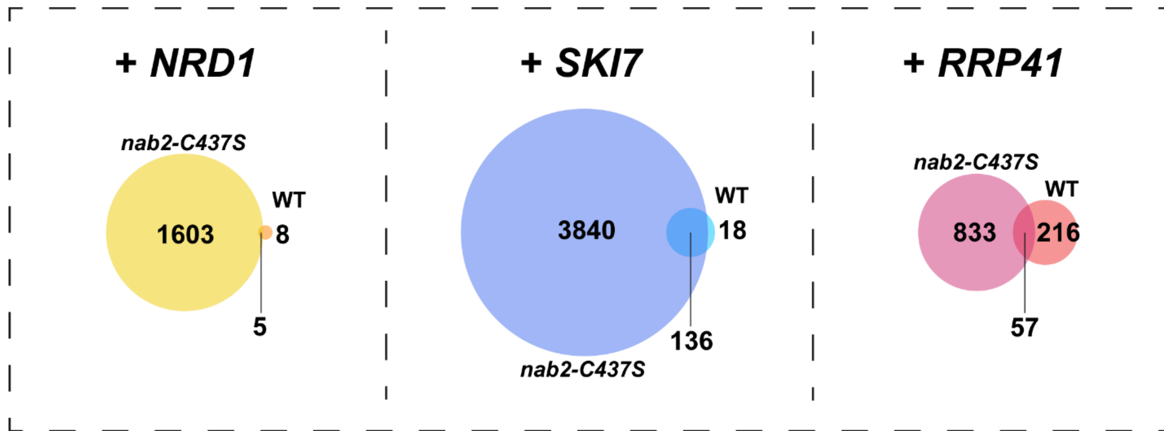


Figure 2-9: Overexpression of suppressor genes in control cells affects minimal genes compared to overexpression in *nab2-C437S* cells. RNA-sequencing was performed on control cells overexpressing suppressor genes. Differential expression analysis was performed on samples compared to control cells overexpressed with empty vector. Gene counts include transcripts with both up and down steady-state levels. Absolute $\log_2$ fold change cut-off set at $\alpha>0.5$. Statistical significance set at $p<0.05$.

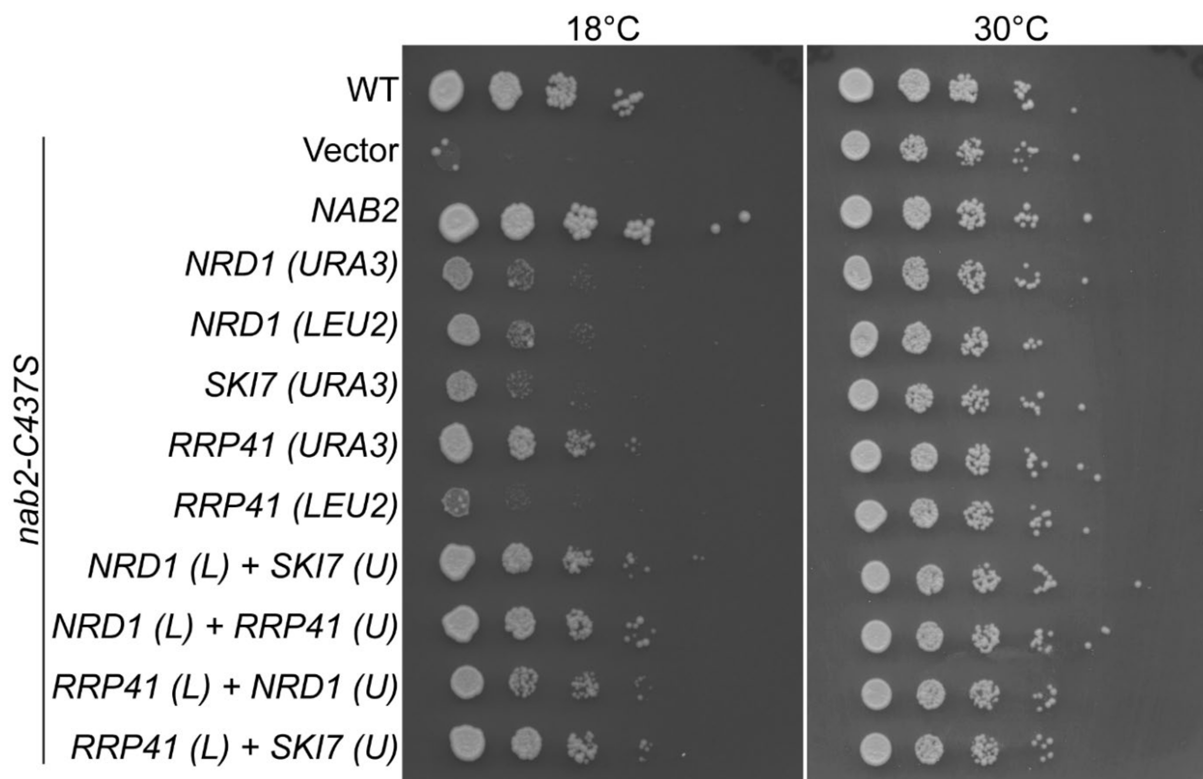
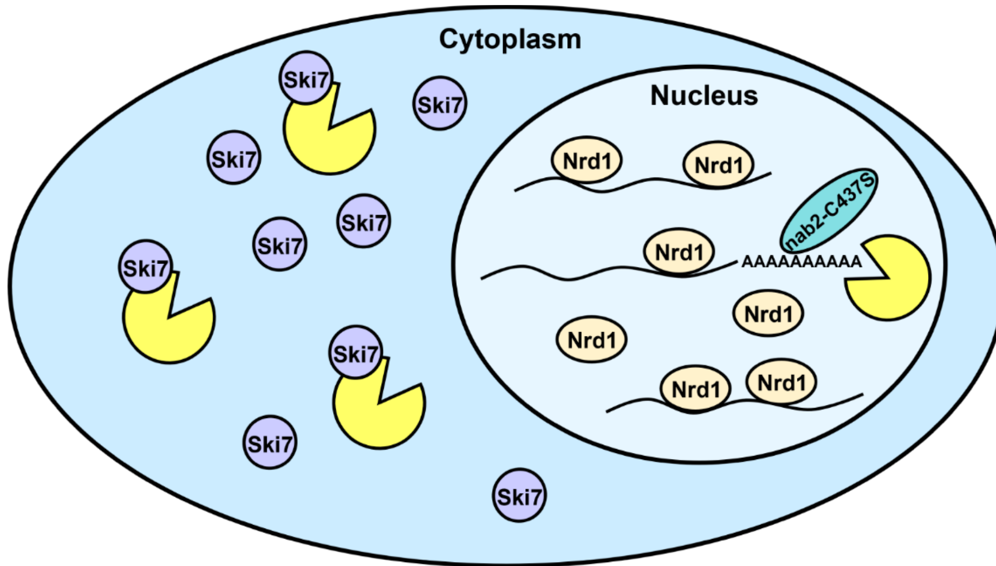
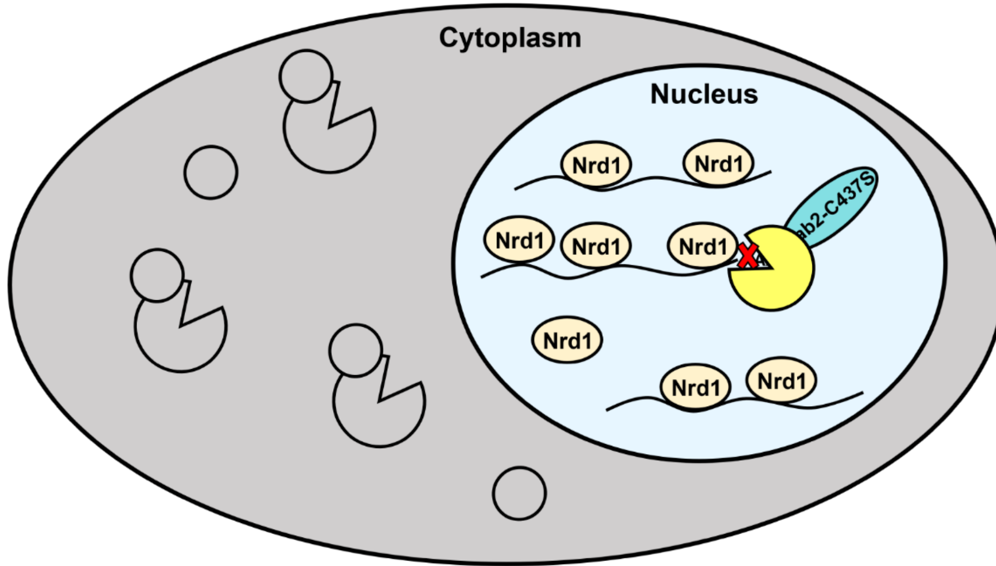


Figure 2-10: Co-overexpression of *NRD1* and *SKI7* improves growth compared to single overexpression but not more than *RRP41* overexpression. *Nab2-C437S* cells were transformed with 2μ plasmids containing *NRD1*, *SKI7*, and/or *RRP41* genes. *URA3* and *LEU2* selective marker genes on plasmids were used to allow selection for two suppressors simultaneously. Cells were grown at 18°C to accentuate subtle growth differences. *U*, *URA3*; *L*, *LEU2*.


Cytoplasm
Nucleus
Ski7
Ski7
Ski7
Ski7
Ski7
Ski7
Ski7
Ski7
Ski7
Nrd1
Nrd1
Nrd1
Nrd1
Nrd1
Nrd1
Nrd1
nab2-C437S
AAAAAAAAAA


Cytoplasm
Nucleus
Nrd1
Nrd1
Nrd1
Nrd1
Nrd1
Nrd1
Nrd1
Nrd1
nab2-C437S

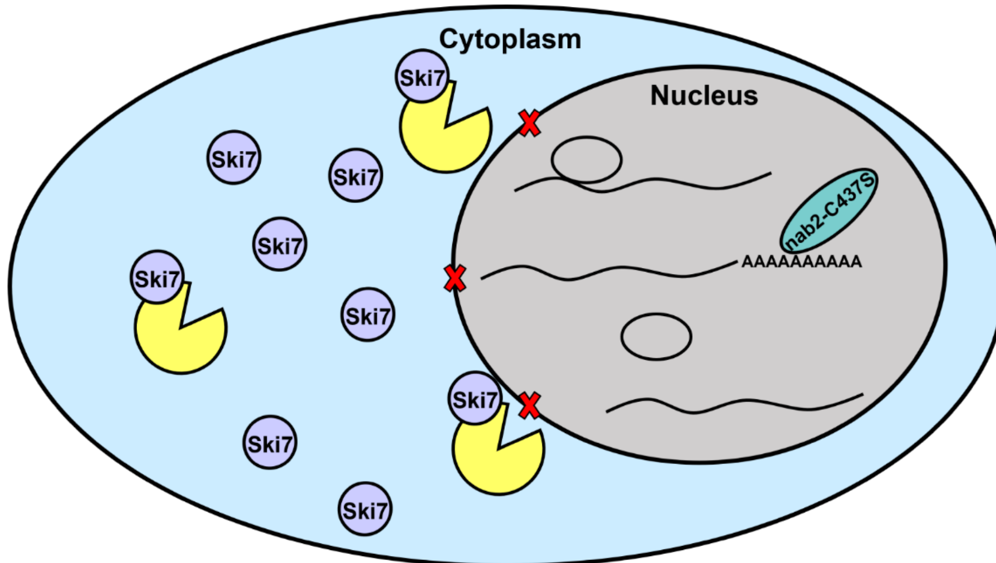

Cytoplasm
Nucleus
Ski7
Ski7
Ski7
Ski7
Ski7
Ski7
Ski7
Ski7
Ski7
nab2-C437S
AAAAAAAAAA

Figure 2-11: Model: Overexpression of RNA exosome cofactors *NRD1* and *SKI7* impair nuclear RNA exosome function. Weakened binding between Nab2-C437S and target RNAs may result in misprocessed RNAs that are quickly degraded by the RNA exosome. Upon overexpression of *NRD1*, Nab2-target RNAs may be protected from degradation by the RNA exosome due to promiscuous binding of Nrd1. Upon expression of SKI7, the RNA exosome may be sequestered in the cytoplasm, and Nab2-target RNAs may be protected from degradation due to decreased nuclear localization of the RNA exosome.

Chapter 3:

The steady-state level of *TLC1*, the RNA scaffold component of *S. cerevisiae* telomerase, is decreased in *nab2-C437S* cells without affecting telomere length or lifespan.

[I generated all data shown in this chapter.]

3.1 Introduction

3.1.1 Telomeres protect chromosome ends from degradation

During DNA replication, the linear ends of chromosomes are vulnerable to degradation due to the end replication problem [134]. The end replication problem refers to the inability of the lagging strand of DNA to be replicated to the end because of the removal of the final RNA primer, leaving a single-stranded DNA overhang. Fortunately, instead of valuable genetic sequences, the very ends of chromosomes are comprised of repetitive DNA sequences [135]. These repetitive TTAGGG sequences together are called telomeres.

3.1.2 Telomerase and TLC1

Telomere length is maintained by telomerase [136-139], which is a holoenzyme comprised of several proteins (Est1, Est2, and Est3) and a structural RNA, TLC1. TLC1 also serves as the template for telomeric DNA. Along with the Est proteins, TLC1 associates with Ku70/Ku80, Pop1/Pop6/Pop7, and the Sm7 ring complex [140, 141], and this telomerase holoenzyme moves to telomere ends to protect them from shortening. TLC1 is the rate-limiting component of telomerase, and a reduction in *TLC1* levels results in a reduction in telomerase activity [142]. Without functional telomerase, telomere ends are rapidly shortened, leading to cellular senescence.

TLC1 exists in two forms in the cell, a long, polyadenylated form and a short form that functions within the telomerase complex. The short, mature form comprises 90% of *TLC1* transcripts, while only 10% are polyadenylated [143]. Polyadenylated *TLC1* is the precursor form of *TLC1* that is exported into the cytoplasm where it associates with

additional proteins. Polyadenylated *TLC1* is then imported into the nucleus where it is processed into the short, mature form [144].

3.2 Results

3.2.1 Telomere maintenance genes have decreased steady-state levels in *nab2-C437S* cells.

In the high-copy suppressor screen described in Chapters 1 and 2, *nab2-C437S* cells showed decreased steady-state levels of a subset of transcripts. Gene set enrichment analysis (GSEA) revealed that significantly decreased transcripts cluster into fourteen gene ontology categories. Two of these categories relate to telomere maintenance (Figure 2-5). From these two categories, three transcripts contribute most substantially to the enrichment score of telomere maintenance. These are *CDC13*, *HSP82*, and *TLC1*. *CDC13* is an essential gene encoding Cdc13/Est4 which negatively regulates telomerase activity [145-147]. *HSP82* encodes a heat shock protein that serves as a chaperone for Cdc13, impacting Cdc13 activity [148]. Finally, TLC1 (telomerase component 1) is the RNA scaffold component of the yeast telomerase holoenzyme [4].

3.2.2 Nab2 plays a role in *TLC1* processing.

As mentioned in Chapter 1, Nab2 binds polyadenosine stretches of RNA. Although the short mature form of *TLC1* comprises the majority of nuclear *TLC1* transcripts, Nab2 likely assists in processing of the polyadenylated precursor. In support of this concept, previous work has shown that polyadenylated *TLC1* levels decrease upon depletion of Nab2 [107]. Due to the impairment of nuclear export after Nab2 depletion, it has been suggested that the effect on *TLC1* could be related to the export defect rather than to processing by Nab2 [144]. However, the decrease in steady-state

levels of *TLC1* in *nab2-C437S* cells, which lack a bulk RNA export defect [48, 49], suggests a more direct role of Nab2 in *TLC1* processing.

The Nab2-C437S protein shows significantly reduced binding to target RNAs compared to wildtype Nab2 [48]. In *nab2-C437S* cells, the immature, polyadenylated form of *TLC1* may be left unprotected and exposed to degradation by the RNA exosome. Degradation of the precursor would result in the export of fewer *TLC1* transcripts to the cytoplasm and, subsequently, fewer transcripts imported into the nucleus for processing into the mature form. This reduction could result in the decreased steady-state level of *TLC1* in *nab2-C437S* cells.

3.2.3 Reduced *TLC1* levels in *nab2-C437S* cells do not result in shorter telomeres or cellular senescence.

Reducing the level of *TLC1* by 50% has been shown to result in shortened telomeres and cellular senescence [142]. In *nab2-C437S* cells, *TLC1* levels decrease by a fold change of 0.32, or log2 fold change of -1.65 when compared to wildtype cells (Figure 3-1). This sharp decrease exceeds the threshold previously reported to result in shortened telomeres [142]. Surprisingly, bulk telomere length, as measured by Southern blotting for telomere ends, does not decrease in *nab2-C437S* cells (Figure 3-2A). Additionally, severely decreased *TLC1* levels should result in cellular replicative senescence. Replicative lifespan was measured by selecting individual colonies from each genotype and streaking onto new plates. Cells were then grown for two days, and individual colonies were again selected, struck out, and grown for two days. This was repeated over twenty times. In concordance with the lack of telomere shortening in

nab2-C437S cells, *nab2-C437S* cells also do not show a replicative senescence phenotype (Figure 3-2B).

To study telomere length in *nab2-C437S* cells, we compared growth and telomere length with wildtype cells. Additionally, we studied both wildtype *NAB2* and *nab2-C437S* cells with either *KU70* or *KU80* deleted to ensure that no alternative telomere maintenance pathway was active. Ku70 and Ku80 play roles in double-stranded break repair and telomere maintenance [149-151]. ∆*ku70/80* double mutant cells have a shortened telomere phenotype, and we tested whether loss of these proteins would exacerbate a phenotype in *TLC1*-deficient *nab2-C437S* cells. Although ∆*ku70/80* cells in both wildtype *NAB2* and *nab2-C437S* cells did show shortened telomeres (Figure 3-2A), cells did not show a senescence phenotype (Figure 3-2B). Furthermore, *nab2-C437S*/∆*ku70/80* cells did not show shorter telomere length than *NAB2/*∆*ku70/80* cells.

3.2.4 Deletion of *RRP6* results in substantially increased levels of *TLC1*.

The RNA exosome plays a crucial role in regulating *TLC1* transcripts [152]. When the gene encoding the RNA exosome cofactor/subunit Rrp6 is deleted, *TLC1* levels increase drastically, even beyond wildtype levels (Figure 3-1). *Nab2-C437S* cells have decreased steady-state levels of *TLC1*, but when *RRP6* is deleted in these cells, *TLC1* levels are restored beyond wildtype levels, albeit not to the same extent as when *RRP6* is deleted in control cells. Surprisingly, impairment of the RNA exosome by overexpression of suppressor genes *NRD1*, *SKI7*, and *RRP41* (Chapter 2) does not rescue *TLC1* steady-state levels (data not shown). This may be due to the lesser extent

of RNA exosome impairment by overexpression versus eliminating an RNA exosome subunit.

3.3 Discussion

Although *TLC1* levels are severely decreased in *nab2-C437S* cells, the expected shortened telomeres and cellular senescence phenotype does not occur. This finding is surprising due to *TLC1* levels in *nab2-C437S* cells decreasing below an established threshold for shortened telomeres [142]. As TLC1 is the rate-limiting component of the telomerase holoenzyme, a reduction in TLC1 should result in a reduction in telomerase complexes and activity, and the lack of this phenotype suggests an alternative method for maintenance of telomere length.

To explain the lack of a deleterious phenotype, we first considered Alternative Lengthening of Telomeres (ALT) as a potential mechanism [153]. In ALT, a small number of cells deficient for one member of the telomerase complex (Est1, Est2, Est3 or TLC1) or with impaired Cdc13 recruitment maintain telomere length and avoid early senescence through a recombination-mediated amplification of telomeric and subtelomeric sequences [154, 155]. In *nab2-C437S* cells, however, there appears to be no deficiency in telomere length maintenance or replicative senescence. If ALT were to explain this phenomenon, only a small fraction of cells would recover.

Potentially, there could be background-specific (W303) variation allowing *nab2-C437S* cells to bypass a *TLC1* requirement. As ∆*ku70/80* deletion cells showed shortened telomeres, this suggests a peculiarity in *TLC1* requirement rather than all elements of telomerase. A lack of replicative senescence upon reduction in *TLC1* levels and/or depletion of Ku70 or Ku80, suggests a potential growth maintenance mechanism that we do not yet understand. Further investigation is merited to identify causative factors for this distinct phenotype.

Finally, the rescue of *TLC1* steady-state levels by Δ*RRP6* but not by overexpression of suppressor genes *NRD1*, *SKI7*, and *RRP41* is surprising, particularly as Nrd1 plays a role in transcription termination of polyadenylated *TLC1* [121, 156]. If the state of NNS function is not critical for suppression or affected by *NRD1* overexpression, however, the relationship between *TLC1* and Nrd1 may be inconsequential in this context. RNA exosome function may be affected by overexpression in a manner that does not affect *TLC1*. Another possibility is that Rrp6 function, in particular, is critical for *TLC1* processing, and overexpression of suppressors may affect core exosome function to a greater extent than Rrp6-specific function. This is an intriguing question whose answer may be found as part of the root mechanism of suppression.

In conclusion, these studies have implications for the mechanism by which Nab2 may regulate *TLC1* and potentially other non-coding RNAs. The studies also reveal a surprising and not yet understood mechanism by which cells mutant for *nab2* maintain telomere length despite a significant decrease in *TLC1* levels.

Figures – Chapter 3:

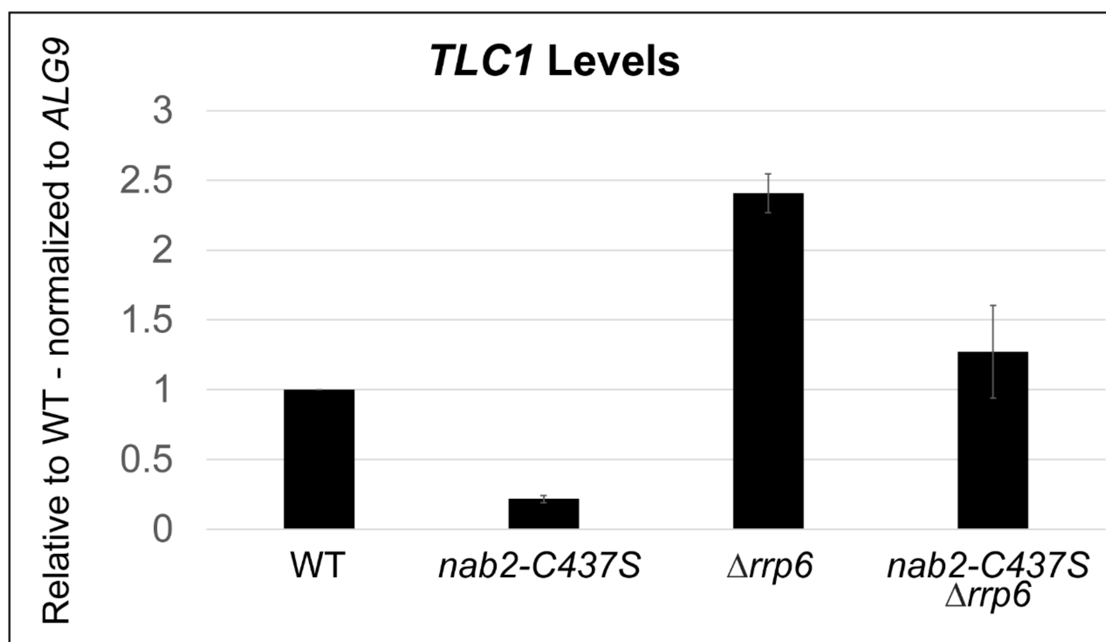
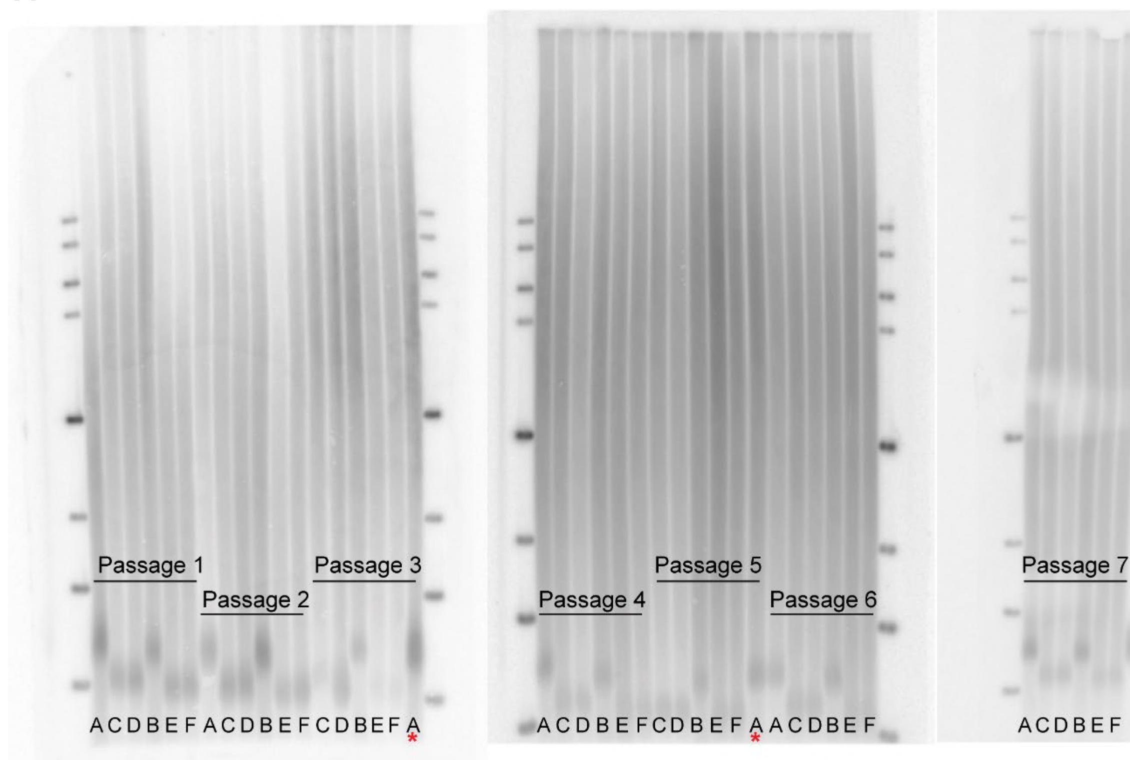


Figure 3-1: *TLC1* levels are decreased in *nab2-C437S* cells, and growth is rescued by deletion of *RRP6*. Using biological triplicates of each sample, *TLC1* levels were measured via qRT-PCR. Comparisons were calculated using the $2^{(-\Delta\Delta CT)}$ method [157]. Statistical significance for pairwise comparisons set at $p<0.05$.


A
Passage 1
Passage 2
Passage 3
A C D B E F A C D B E F C D B E F A
*
Passage 4
Passage 5
Passage 6
A C D B E F C D B E F A A C D B E F
*
Passage 7
A C D B E F

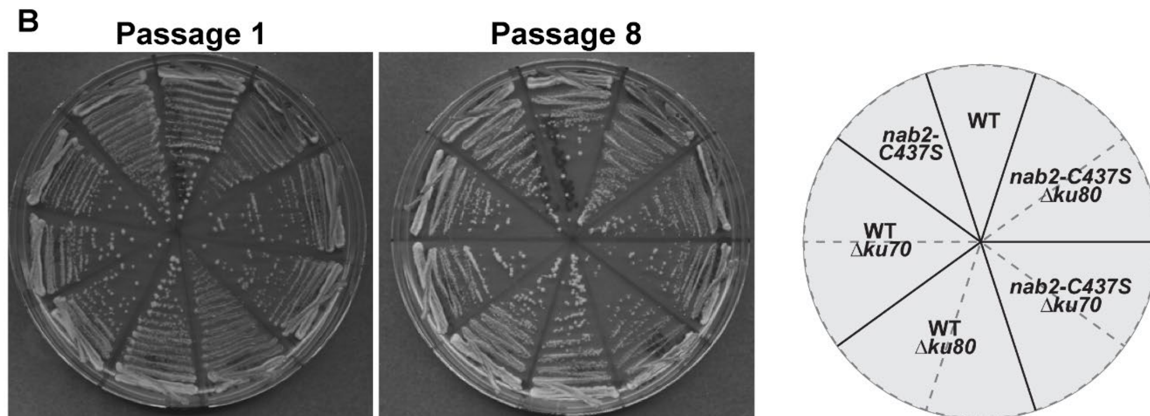

B
Passage 1
Passage 8
WT
nab2-C437S
nab2-C437S Δku80
WT Δku70
nab2-C437S Δku70
WT Δku80

Figure 3-2: *Nab2-C437S* cells do not show shortened telomeres nor cellular senescence. Passage numbers refer to the number of times cells were picked from previous plates of each sample to either grow on solid media plates for 2 days or grow in liquid media for subsequent DNA extraction. (A) Southern blotting was performed on DNA from multiple passages of each sample to measure bulk telomere length. Shorter telomeres appear further down on the blots. Typical telomere length is represented by the wildtype sample for each passage. [A = WT, B = Δ*ku70*, C = Δ*ku80*, D = *nab2-C437S*, E = *nab2-C437S* Δ*ku70*, F = *nab2-C437S* Δ*ku80*]. Red asterisks indicate samples run out of sequence for a particular passage, with A-C-D-B-E-F being the typical and intended order. (B) Strains were struck out on solid media plates many times to detect replicative senescence. The orientation of samples on plates matches the diagram to the right.

Chapter 4:

General Discussion

4.1 Recap and implications of this study

Prior to this study, the relationships between Nab2 and RNA exosome cofactors Nrd1 and Ski7 were not well-characterized. The nuclear RNA exosome is important for degrading improperly processed RNAs, including those that may be misprocessed due to impairment of Nab2 function in *nab2-C437S* cells; however, the degree of importance of specific RNA exosome cofactors in a mutant *nab2* context had not been explored. The identification of RNA exosome cofactors as suppressors of the *nab2-C437S* cold-sensitive growth defect in a previous study (Mills-Lujan et al, in preparation) set the stage for a more detailed study of the critical functions and interplay of RNA exosome cofactors, the RNA exosome, and RNA binding protein Nab2.

This study examined the critical RNA exosome cofactor interactions required for suppression of the *nab2-C437S* growth defect. Our findings shed light on which cofactor functions promote suppression and how these functions relate to RNA exosome function. We also analyzed how the transcriptome is impacted by mutant *nab2* and further impacted by overexpression of RNA exosome cofactors. These findings shed light on how RNA exosome cofactor expression impacts, not only steady-state levels of RNAs in relation to Nab2, but how RNAs are processed, as well. By studying the effects of RNA exosome cofactors on RNA exosome function in a mutant *nab2* context, we have highlighted the precarious balance of RNA processing, RNA protection, and RNA degradation in post-transcriptional control of gene expression.

4.2 Transcriptomic changes occurring from suppressor overexpression are broad but subtle.

After identifying transcripts with altered steady-state levels from the RNA-seq experiment described in Chapter 2, we sought to validate these expression changes using quantitative real-time PCR (qRT-PCR). Optimal transcripts to validate were those found to decrease in *nab2-C437S* cells and show an increase or restoration to normal levels upon overexpression of suppressors. RNA-seq BAM files were uploaded into Integrated Genome Viewer (IGV) for closer examination of reads along the genome to visually assist in target RNA selection for this analysis.

Ribosomal protein gene *RPS22B* is one of the most decreased RNAs in *nab2-C437S* cells. Ribosomal protein genes comprise a significant portion of transcripts affected by the *nab2-C437S* mutation. Similarly, *SNR44*, a snoRNA gene encoded within the second intron of *RPS22B*, shows a sharp decrease in steady-state level in *nab2-C437S cells*. *RPS22B* and *SNR44* levels increase upon *NAB2* expression to rescue the nab2-C437S cells, as expected. Overexpression of *NRD1*, *SKI7*, or *RRP41*, however, show significant increases in steady-state levels of *SNR44* compared to *nab2-C437S* cells, while *RPS22B* only shows a mild increase when any of the suppressors identified are overexpressed. qRT-PCR results validated the rescue of steady-state levels by *NAB2*; however, neither *RPS22B* nor *SNR44* steady-state level increases could be validated using qRT-PCR, despite trying multiple primers. This is surprising due to the significant increase in *SNR44* levels shown in the RNA-seq data. We can only speculate that there is altered processing of these transcripts that is not revealed by these short read RNA-Seq techniques. An approach using long-read RNA-Seq might

help to illuminate some of the changes in these target transcripts that occur when these suppressors, which clearly rescue growth defects, are overexpressed.

For the majority of transcripts with decreased steady-state levels in *nab2-C437S* cells, suppressor overexpression only results in a mild to moderate increase. *SKI7*, the suppressor with the most dramatically distinct transcriptomic profile (Figure 2-4), shows the largest individual RNA steady-state level increases and decreases compared to *nab2-C437S* cells. Due to the moderate steady-state level changes found for suppressors, validation of the small changes by qRT-PCR was very challenging .

4.3 *Nab2-C437S* cells show RNA processing defects.

A recent study revealed that rapid and complete depletion of Nab2 can cause transcriptional readthrough in a subset of RNAs [158]. Our study utilizes *nab2* mutant cells, *nab2-C437S*, rather than Nab2 depletion, but we wanted to test whether *nab2-C437S* cells also show transcriptional readthrough of RNAs. IGV reads from the RNA-seq data and unpublished work from our lab show that *nab2-C437S* cells have a very modest transcriptional readthrough defect, albeit not as strong as the defect seen in cells depleted of Nab2.

After confirming read-through defects in *nab2-C437S* cells, the next step was to determine whether overexpression of suppressors could mitigate the readthrough phenotype. One of the suppressors, Nrd1, is directly involved in transcription termination of short RNAs. This raises the possibility that overexpression could affect a readthrough phenotype. Upon examination of target RNAs through RT-PCR, we found that suppressors do not restore precise transcription termination (data not shown). In

fact, rather than improving transcription termination, *NRD1* overexpression appears to increase readthrough in target RNAs.

4.4 Overexpression of *NRD1* results in RNA processing defects.

While attempting to validate transcripts identified in our RNA-seq experiment using qRT-PCR, we measured an RNA called *CMC4*. The RNA-seq data showed a decreased steady-state level of *CMC4* in *nab2-C437S* cells with an increased level upon overexpression of *SKI7*. Both *NRD1* and *RRP41* overexpression decreased *CMC4* levels further. Using qRT-PCR, we measured steady-state levels of *CMC4* and found a significant change only upon *NRD1* overexpression in *nab2-C437S* cells. This result was surprising due to the decrease shown in the RNA-seq data.

Upon examining *CMC4* reads in IGV, we noticed an increased abundance of intronic *CMC4* reads (Figure 4-1A/B). To determine if a splicing or processing error could result in the contrary qRT-PCR result of increased reads upon *NRD1* overexpression, an additional forward primer was designed to span the exon-exon boundary of *CMC4*, which would exclude improperly spliced transcripts. The original forward primer was designed completely in the first exon, allowing inclusion of both properly and improperly spliced products in the steady-state level count. Intriguingly, qRT-PCR results showed a decrease in *CMC4* reads when intronic reads were excluded. This suggests that *NRD1* overexpression results in a transcript processing defect. To validate this result, reverse-transcriptase PCR (RT-PCR) products using both primer *CMC* primer pairs were compared, and intron inclusion was noted for *CMC4*.

Additional transcripts with possible processing defects were identified using IGV. These transcripts include *RPS9A*, *RPL7B*, *TUB3*, and *ERV14*. *RPS9A* and *RPL7B* both display increased intronic reads when *NRD1* is overexpressed in *nab2-C437S* cells compared to both *nab2-C437S* and control wildtype samples (Figure 4-1C/D). Interestingly, overexpression of *NRD1*, *SKI7*, or *RRP41* show increased 3' end reads of *TUB3* (Figure 4-2A/B), but intronic reads do not show an increase. *ERV14* shows increased 5' end reads for all suppressor samples (Figure 4-2C). Measurement of reads using RT-PCR will need to be performed to validate these results. However, as suppressors increase processing defects and transcriptional readthrough rather than restoring processing in *nab2-C437S* cells, processing defects are likely only a byproduct of *NRD1*, *SKI7*, and/or *RRP41* overexpression and not central to a mechanism of suppression. As such, further validation and characterization of the defects is inconsequential to understanding the relationship of Nab2 to the suppressors.

It is worth noting that the persistent and sometimes exacerbated processing defects seen for *nab2-C437S* cells and *nab2-C437S* cells with overexpression of suppressors, may actually be the result of mutant *nab2* exclusively and not due to misprocessing by suppressors. By impairing RNA exosome function through overexpression of *NRD1*, *SKI7*, or *RRP41*, transcripts misprocessed by mutant Nab2 may persist in the nucleus without degradation by the exosome. This is a possible explanation, but it could also be a partial explanation. Some transcripts may be further misprocessed when *NRD1*, *SKI7*, or *RRP41* is overexpressed due to aberrant RNA exosome cofactor activity. The misprocessing identified exclusively upon *NRD1* overexpression, for example, could be an artifact of aberrant Nrd1 activity, and, thus,

account for the *NRD1* overexpression specificity. Further analysis could distinguish the processing defects perpetrated by mutant Nab2 and subsequently revealed by RNA exosome impairment versus processing defects caused by aberrant suppressor activity.

4.5 *AZF1* overexpression suppresses the *nab2-C437S* cold-sensitive growth phenotype.

Besides the RNA-exosome related suppressors, *AZF1*, or Asparagine-Rich Zinc Finger Protein 1, was also identified as a suppressor of *nab2-C437S* cold-sensitive growth in the high-copy suppressor screen. Azf1 is a yeast-specific transcription factor that activates transcription of genes involved in multiple cellular functions. Depending on cellular conditions, Azf1 activates genes involved in either cell wall maintenance or in cell growth and metabolism [159]. Overexpression of Azf1 increases cell growth during stress [160]. Overexpression has also been shown to suppress a mutation in a mitochondrial RNA polymerase gene [161]. Rather than through a direct interaction with Nab2, Azf1 may suppress the cold-sensitive growth defect of *nab2-C437S* cells through an indirect mechanism. Azf1 may activate or inactivate a gene or set of genes that are able to increase the growth rate of *nab2-C437S* cells. Overexpression of *AZF1* may affect metabolic functions that mitigate the growth defect. A direct effect on *NAB2* or interaction with the Nab2 protein is unlikely.

Conversely, the RNA exosome interacts with Nab2, and RNA exosome-related suppressors likely suppress the *nab2-C437S* growth defect in a more direct fashion. However, a known relationship between Nab2 and the RNA exosome does not necessarily preclude a more indirect method of suppression. One possibility is that the

RNA exosome-related suppressors may suppress the growth defect through a more general stress response.

4.6 The environmental stress response does not explain altered steady-state transcript levels by suppressors.

One possible explanation for altered gene expression upon overexpression of the suppressor genes identified here, *NRD1*, *SKI7*, and *RRP41*, is that these could mitigate negative effects of an environmental stress response (ESR) induced by cold-exposure of cells by affecting expression of the approximately 300 up-regulated and 600 down-regulated genes involved in this response [162]. Simply put, the ESR up-regulates genes involved in cellular pathways that provide dynamic adaptation to stressors and down-regulates genes involved with cell growth. Ultimately, cell growth is slowed to allow recovery. By subjecting *nab2-C437S* cells to cold temperatures, the ESR could be triggered, resulting in slow cell growth. RNA exosome-related suppressors could rescue growth at cold temperatures by increasing expression of genes involved with growth. To test this idea, the genes involved in the ESR were compared to the lists of genes affected by suppressors. We found that genes affected by overexpression of suppressors did not closely overlap with genes induced or repressed by the ESR (data not shown), suggesting that the mechanism of suppression may not be a general environmental stress response.

4.7 Potential mechanism of suppression by Mtr4

As described in Chapter 2, the high-copy suppressor screen yielded *NRD1*, *SKI7*, *NOP8*, *RRP41*, and *RRP42* as suppressors of the *nab2-C437S* cold-sensitive growth phenotype. After verifying these suppressors (Figure 2-1A), a panel of other nuclear and cytoplasmic RNA exosome cofactors was tested for suppression to determine whether RNA exosome cofactors broadly suppress the *nab2-C437S* cold sensitive growth. As shown in Figure 2-1B, *MTR4* was also identified as a suppressor of the *nab2-C437S* growth phenotype. With the identification of *MTR4* as a suppressor from this small candidate panel, it was not the focus of the study; however, following up on this result could reveal greater insight into how impairment of RNA exosome function rescues *nab2-C437S* cold-sensitive growth.

Mtr4 is the helicase component of the Trf4/5-Air1/2-Mtr4 (TRAMP) complex [163]. Mtr4 contains five key domains (Figure 4-3). At the N-terminus, it contains a disordered region where it binds to Rrp6-Rrp47 [133, 164]. It contains two helicase domains (RecA-1 and RecA-2) required for helicase and ATPase activity of Mtr4. Additionally, in humans, it is within these helicase domains that MTR4 interacts with RNA exosome cofactor MPP6 [165]. The arch domain, contained within a structural domain, is required for RNA binding, as well as RNA processing and degradation [166-168]. The final domain is a ratchet helix domain that is both structural and contributes to the unwinding rate of target RNAs [168, 169].

As was done for Nrd1 and Ski7, testing the requirement for each domain of Mtr4 could elucidate the necessary interactions for suppression. If the N-terminal domain was required, it would indicate the necessity of the interaction between Mtr4 and Rrp47-

Rrp6. If the RecA-1/2 helicase domains were required, this would indicate a requirement for helicase activity and potentially an interaction with Mpp6. The requirement for the arch domain could indicate the requirement for RNA binding or further RNA processing. Finally, the necessity of domain 4 could indicate the importance of maintaining the unwinding rate of target RNAs, while necessity for the structural domains could indicate structural integrity as pivotal. To parse out required roles and activities of Mtr4, mutations and/or deletions that impair these functions would need to be generated. The possibility remains that multiple domains could be required. Furthermore, uncovering the functions and interactions of Mtr4 is an active area of investigation and would require further study to understand the interplay of various domains for particular functions.

Mtr4 also interacts with the other subunits of the TRAMP complex, Trf4/5 and Air1/2 [170]. Testing the requirement for interactions with the rest of the complex would reveal whether TRAMP complex function is required for suppression or whether suppression by *MTR4* is independent of TRAMP. Additionally, overexpression of *MTR4* could result in altered stoichiometry of the TRAMP complex, ultimately affecting TRAMP-related activities and processing roles.

If, like Nrd1, the RNA binding function of Mtr4 was required for suppression, this could indicate a potentially similar mode of suppression for *NRD1* and *MTR4*. Perhaps by binding target RNAs more strongly, Mtr4 could outcompete other proteins for binding. Among other interactions, Mtr4 competes with the RNA exosome cofactor/subunit Rrp6 for binding and processing of target RNAs [116], and this competition could be critical and potentially affected by *MTR4* overexpression. Ultimately, uncovering the domains

and interactions of Mtr4 required for suppression of the *nab2-C437S* growth defect could shed light on a potentially critical interplay of Mtr4 and Nab2 activities. In addition to uncovering how *MTR4* genetically interacts with *NAB2*, findings could reveal functions of the RNA exosome that are critical in relation to Nab2 function.

4.8 Potential mechanism of suppression by Nop8

Additional work could also be performed to distinguish the required domains and interactions of Nop8 for suppression. Nop8 contains an RNA binding domain in its N-terminal region and a coiled-coil domain in its C-terminal region (Figure 4-4) [114]. Through its N-terminal region, Nop8 binds RNAs, including 5.8S rRNA. Nop8 interacts directly with multiple proteins through the coiled-coil domain, including Dbp6 [171, 172], Nip7 [113], and Rrp6 [114]. Dbp6 is an RNA helicase, which, along with three other proteins, forms a complex with Nop8 that is involved in ribosomal subunit biogenesis. The interaction between Nop8 and Nip7 affects the interaction of Nip7 with pre-ribosome complexes. Nip7 also interacts directly with RNA exosome subunit Rrp43, providing a connection between Nop8 and the core RNA exosome. Finally, Nop8 interacts directly with Rrp6 and, through this interaction, affects core RNA exosome function.

Strikingly, Nop8 inhibits RNA exosome activity in a concentration-dependent manner [114], in opposition to Nop53, another RNA exosome cofactor, which enhances RNA exosome activity [173]. This finding strongly suggests a mechanism of suppression of the *nab2-C437S* growth defect through RNA exosome impairment. Domain analysis of Nop8 could reveal the requirement of RNA binding and/or

interaction with binding partners for suppression. Determining whether interaction with the RNA exosome is required for Nop8-mediated suppression of *nab2-C437S* cells, either directly through Rrp6 or indirectly through Nip7, which interacts with Rrp43, could also reveal a more specific mechanism of RNA exosome impairment by Nop8. Such data could add to the picture that impairing the function of the RNA exosome in a specific manner is sufficient to rescue the cold-sensitive growth of *nab2-C437S* cells.

4.9 A potential role for Rrp6 in suppression

All four RNA exosome cofactors identified as suppressors of the *nab2-C437S* cold-sensitive growth phenotype functionally or directly interact with the RNA exosome cofactor/subunit Rrp6. The possibility that disrupting Rrp6 function is the crux of suppression by RNA exosome cofactors is an exciting direction for current discussion and future study.

Nrd1 has multiple functional interactions with Rrp6. Firstly, Nrd1 and Rrp6 compete for interaction with the nuclear RNA exosome cofactor Mpp6 [174]. This competition may determine whether transcripts terminated by Nrd1 are processed by the RNA exosome catalytic subunit Dis3 or by Rrp6. This interaction occurs through the C-terminal interacting domain (CID) of Nrd1, which also mediates interactions between Nrd1 and Trf4, Polymerase II (Pol II), and the NNS complex subunit Sen1. As discussed in Chapter 2, Nrd1 CID is dispensable for suppression of the cold-sensitive growth of *nab2-C437S* cells (Figure 2-3D). As such, the interactions between Nrd1 and Mpp6, Trf4, Pol II, and Sen1 are not required for suppression. This indicates that the

competition between Nrd1 and Rrp6 for interaction with Mpp6 or possible out-competition by Nrd1 is not responsible for suppression [119].

Another interaction between Nrd1 and Rrp6 is in a messenger ribonucleoprotein particle (mRNP) quality control pathway that also involves Trf4 and Nab3 [118]. In this pathway, improperly processed or packaged mRNPs are retained in the nucleus rather than exported. The mRNAs are then slated for degradation by the RNA exosome. The quality control pathway components Nrd1, Nab3, Rrp6, and Trf4 are recruited to these mRNAs [119]. In the process of recruitment to misprocessed mRNAs, these components become underrepresented along noncoding RNAs where they would otherwise be present for further processing. Additionally, analysis of binding of these components along misprocessed mRNAs showed that binding by Nrd1 is pervasive along transcripts while Nab3, Rrp6, and Trf4 show more discrete binding. Upon overexpression, Nrd1 could potentially preclude binding of these other components by binding misprocessed mRNAs. Simultaneously, an excess of Nrd1 could pervasively bind the short noncoding RNAs it normally binds during transcription termination and disrupt the termination process.

As discussed in Chapters 1 and 2, the *nab2-C437S* mutation results in extended poly(A) tails and the Nab2-C437S protein shows reduced binding affinity for polyadenosine RNAs. These defects could result in mRNP processing defects, as in a recent study [119], which could result in recruitment of quality control components to those misprocessed mRNAs. In *nab2-C437S* cells, it is likely that Nrd1 is recruited from ncRNAs to mRNAs. Due to the pervasive nature of Nrd1 binding, upon overexpression, Nrd1 could potentially preclude binding of Nab3, Trf4, and Rrp6 by compactly binding

misprocessed mRNAs. Simultaneously, a preponderance of Nrd1 due to overexpression could also result in pervasive binding of noncoding RNAs.

As shown in Figure 2-3D, the only domain in Nrd1 that is required for suppression of *nab2-C437S* cells is the RNA binding domain. Our results demonstrate that this domain is necessary for this suppression, but we have not tested whether the Nrd1 RNA binding domain is sufficient for suppression. Whether the suppression is due to Nrd1 outcompeting other proteins for binding to target RNAs has not yet been determined, but this does raise the possibility that *NRD1* overexpression could affect at least one aspect of Rrp6 function.

In vitro work has shown that Nop8 interacts with Rrp6 through the Nop8 C-terminal domain [114]. In contrast to RNA exosome activation by Nop53, Nop8 inhibits the Rnase activity of the RNA exosome in a concentration-dependent manner. This inhibitory effect depends largely on Rrp6 interaction with the RNA exosome complex [114]. The C-terminal domain of Nop8 is essential; however, interruption specifically of the Interaction between Nop8 and Rrp6 in the context of *nab2-C437S* cells could define a requirement for this interaction for suppression. As discussed briefly in Chapter 2, the concentration-dependent inhibition of the RNA exosome by Nop8 provides further evidence to support the model that RNA exosome inactivation is the mechanism by which suppressors rescue growth of *nab2-C437S* cells.

Rrp6 is a nuclear subunit and cofactor of the RNA exosome. Ski7 is a cytoplasmic RNA exosome cofactor. Functions of the cytoplasmic RNA exosome are largely independent of nuclear RNA exosome cofactors. Likewise, function of the nuclear RNA exosome is largely independent of cytoplasmic RNA exosome cofactors.

With Rrp6 and Ski7 interacting with the RNA exosome in separate compartments, it seems that *SKI7* overexpression should have little effect on nuclear RNA exosome function or have little relationship to Rrp6. However, as mentioned in Chapter 2, in vitro Ski7 can outcompete Rrp6 for binding to the RNA exosome [128]. Both cofactors bind RNA exosome structural subunits Csl4, Mtr3, and Rrp43. Upon overexpression of *SKI7*, it is possible that Ski7 binds an increased proportion of RNA exosome complexes or subunits and competes with Rrp6 for binding.

The RNA exosome must be imported into the nucleus to perform the essential nuclear functions of the complex; however, the mechanism of nuclear import has not been fully elucidated. One possibility is that Rrp6, which contains a classical nuclear localization sequence, assists in recruitment of the complex into the nucleus by binding to Csl4, Mtr3, and Rrp43. If Rrp6 is required for recruitment, the overexpression of *SKI7* could not only disrupt binding between Rrp6 and the RNA exosome but also impair nuclear import of the complex. This hypothesis fits with the model that disrupting some aspect of Rrp6 function is central to the mechanism(s) of suppression of the *nab2-C437S* cold-sensitive growth phenotype by RNA exosome cofactors.

Alternatively, the RNA exosome could be imported into the nucleus in an Rrp6-independent manner. If this is the case, the possible competition between Ski7 and Rrp6 for binding with the RNA exosome may be irrelevant to RNA exosome function and the mechanism of suppression. Instead, overexpression of *SKI7* may sequester the RNA exosome in the cytoplasm. This sequestration could impair nuclear RNA exosome function by preventing adequate import of the complex into the nucleus. Sequestration could also affect cytoplasmic RNA exosome function by increasing the amount of RNA

exosome complexes in the cytoplasm and could subsequently enhance cytoplasmic RNA exosome function.

4.10 Concluding remarks and future directions

This study builds on previous work and provides further insight into the relationship between Nab2 and the RNA exosome. A previous study (Mills-Lujan et al., in preparation) first provided evidence that impairment of RNA exosome function can rescue the cold-sensitive growth defect of *nab2-C437S* cells. The study further suggested that overexpression of the RNA exosome subunits Rrp41 and Rrp42 impairs RNA exosome function and rescues the cold-sensitive growth of *nab2-C437S* cells.

The current study characterized the relationship of Nab2 with the RNA exosome cofactors Nrd1 and Ski7. Like Rrp41 and Rrp42, overexpression of these cofactors suppresses the cold-sensitive growth of *nab2-C437S* cells. This study provides evidence that overexpression of *NRD1* and *SKI7* impairs RNA exosome function. By comparing differential expression profiles, overlapping sets of transcripts and GO terms between RNA exosome cofactor and RNA exosome subunits have been identified. The high degree of overlap reveals similar transcriptomic effects resulting from overexpression of *NRD1*, *SKI7*, and *RRP41*. This, in turn, suggests that, like subunit overexpression, cofactor overexpression impairs RNA exosome function.

Because cofactors play auxiliary roles with the RNA exosome, overexpression is unlikely to disrupt the stoichiometry of the core complex, as has been suggested for *RRP41* and RRP42 overexpression. Instead, cofactor overexpression may impair RNA exosome function by disrupting RNA exosome access to target RNAs or by altering

localization of the complex. These disruptions to RNA exosome function may be coordinated through disruption of Rrp6 function in relation to the core RNA exosome.

This study also uncovered the RNA exosome cofactor gene *MTR4* as a high-copy suppressor of the *nab2-C437S* growth defect. The discovery of this suppressor, along with previously identified suppressor, *NOP8*, also provides evidence that overexpression of these RNA exosome cofactors may impair the function of the complex, potentially through disruption of Rrp6 activity. Nrd1, Ski7, Nop8, and Mtr4 all have critical direct or competitive relationships with Rrp6. The specificity of these cofactors as suppressors can help elucidate critical functions of the RNA exosome and RNA processing that are coordinated through careful balance of Nab2 and RNA exosome activity.

Further characterization of these cofactors as *nab2-C437S* suppressors and their critical effects on RNA exosome function is an area of active study. Analysis of domains and interactions of Mtr4 and Nop8 required for suppression will provide more precise insight into how overexpression of these cofactors impairs RNA exosome function. These analyses could provide evidence for or against a shared underlying mechanism of suppression involving Rrp6. Performing RNA-sequencing on these samples alongside the previously identified suppressors, would provide insight into whether overexpression of *NOP8* and/or *MTR4* affects similar sets of transcripts as the other suppressors. Additionally, analyzing the effects of cofactor overexpression on rRNA levels could provide insight into whether rRNAs are affected and how this compares to the effects of Rrp6 impairment or depletion.

Another experiment of interest is studying the localization of RNA exosome subunits upon *SKI7* overexpression. This could shed light on how Ski7 potentially affects nuclear RNA exosome function. Specifically disrupting or enhancing cytoplasmic RNA exosome function could also reveal whether suppression is reliant on impairment of the nuclear RNA exosome specifically or if cytoplasmic RNA exosome activity is critical for mitigating the growth defect.

Nab2 plays important roles in processing and regulation of poly(A) RNAs. However, not all mRNAs are impacted by Nab2 impairment. Previous work has revealed m6A methylation as a potential mechanism for selectivity [33]. In this study, we identified transcripts affected by the *nab2-C437S* mutation and found that the subset of affected RNAs were largely decreased. These decreased transcripts clustered into only 14 significantly down GO categories. These categories pertain to ribosomal subunits and biogenesis, polymerase activity, and telomere maintenance. However, we have yet to elucidate why these particular RNAs are decreased. Further analysis of this subset of transcripts could provide insight into additional commonalities of RNAs that lead to Nab2 targeting.

Of the 14 GO categories significantly down in *nab2-C437S* cells, *NAB2* overexpression rescues all 14 to some extent. Overexpression of *NRD1*, *SKI7*, or *RRP41*, however, does not rescue all the downregulated categories, but several categories are rescued. Notably, the three categories related to ribosome subunits and biogenesis are all rescued by both *NRD1* and *RRP41* overexpression. This rescue suggests a possible effect on translation, and, ultimately, a potential mechanism of suppression. *SKI7* overexpression, which does not rescue these categories, appears to

have distinct transcriptomic effects. Because *SKI7* overexpression has both nuclear and cytoplasmic effects, there is a possibility that changes in various cytoplasmic processes may mask otherwise apparent alterations in levels of RNAs resulting from nuclear RNA exosome impairment. Following up on translational effects resulting from the *nab2-C437S* mutation and subsequent overexpression of *NRD1* and *RRP41* is an exciting direction for future studies.

This study has characterized the genetic interactions between RNA exosome cofactors and Nab2. Nab2 is conserved in humans as ZC3H14, as described in Chapter 1 and mutations in *ZC3H14* cause an inherited form of intellectual disability [23]. Many RNA exosome cofactors are also conserved and have been implicated in human diseases [82]. By identifying and studying the interactions between Nab2 and RNA exosome cofactors in budding yeast, we seek to provide insight into how RNA exosome cofactors and Nab2 may function together in humans. More specifically, characterization of these relationships could potentially provide insight into how mutations in *ZC3H14* cause neurological disease and how RNA exosome cofactors may play pivotal roles in regulating context-specific effects of ZC3H14 and ZC3H14 dysfunction.

Figures – Chapter 4:

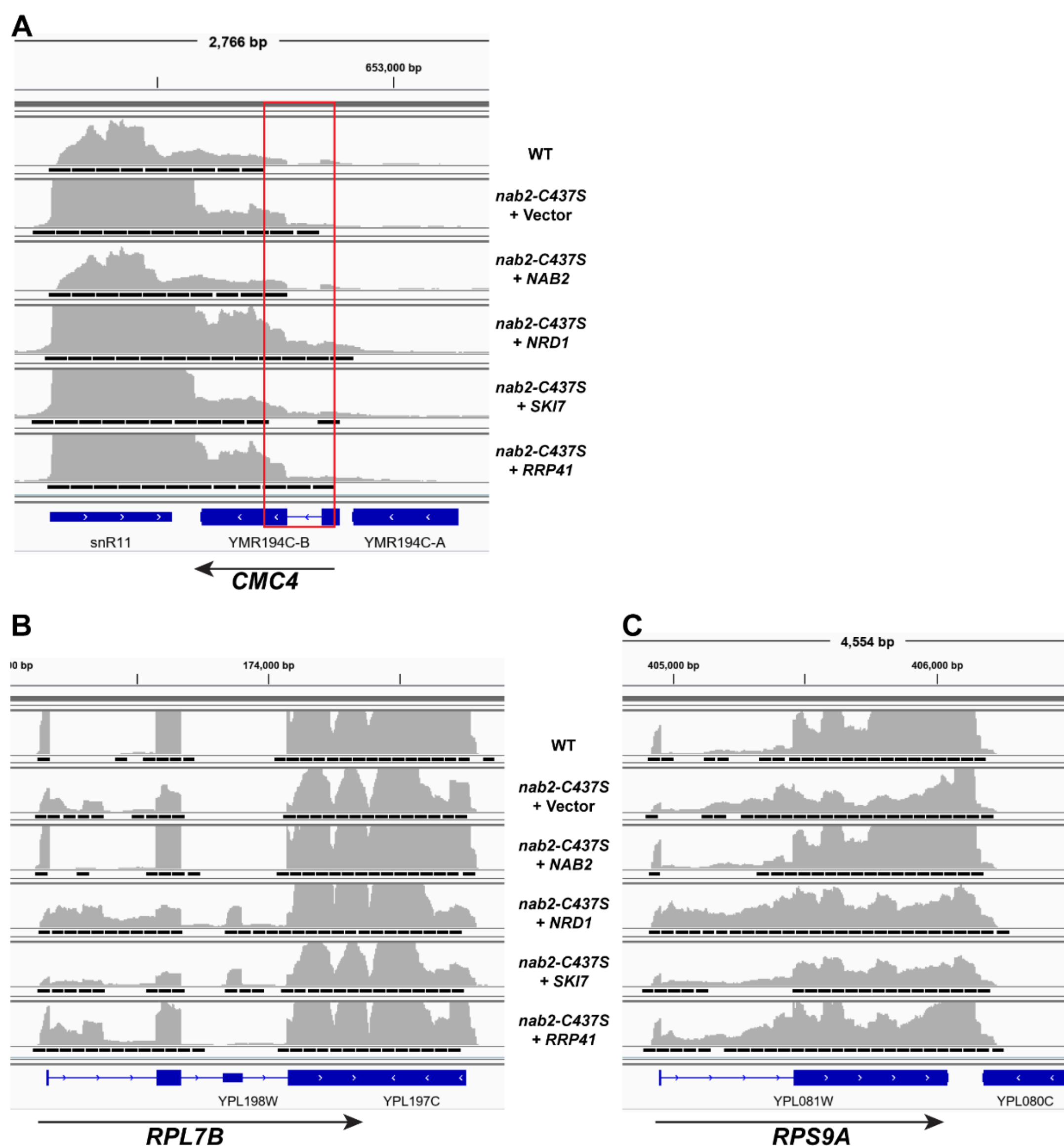


Figure 4-1: Overexpression of suppressors results in increased intronic reads in a subset of transcripts. RNA-seq datasets were imported into IGV, and screenshots were taken, showing abnormal reads. All sample ranges are identical per individual panels. (A) *CMC* shows increased intronic reads when NRD1 is overexpressed. (B-C) Overexpression of *NRD1* or *RRP41*, but not *SKI7*, results in increased intronic reads for *RPL7B* and *RPS9A*.

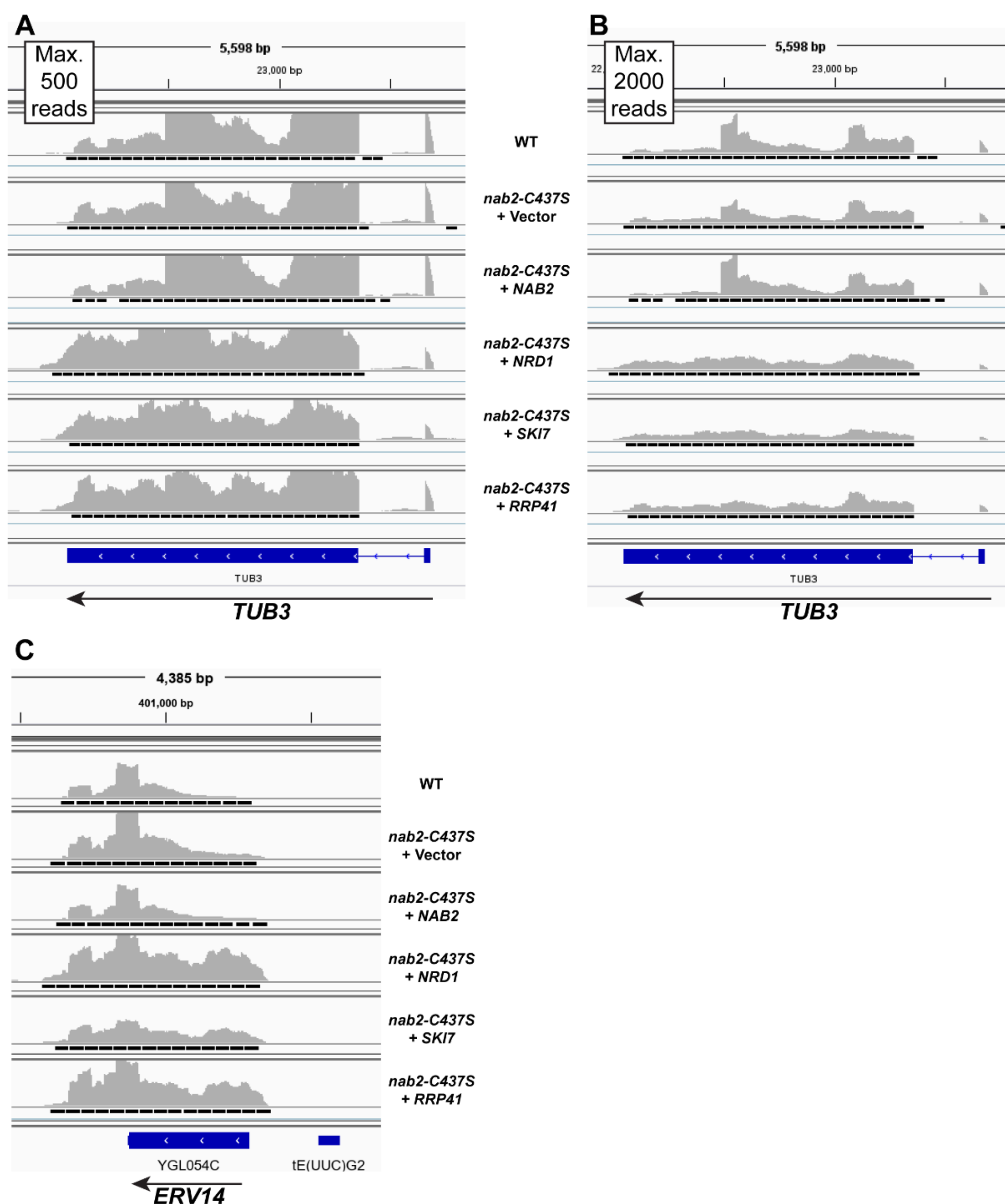


Figure 4-2: Overexpression of suppressors results in 5' and/or 3' end processing defects in a subset of transcripts. RNA-seq datasets were imported into IGV, and screenshots were taken, showing abnormal reads. All sample ranges are identical per individual panels. (A-B) *TUB3* shows increased 3' end reads upon overexpression of *NRD1*, *SKI7*, or *RRP41*, which is not due to increased levels of the entire transcript. (C) *ERV14* shows increased 5' end reads upon overexpression of suppressors.

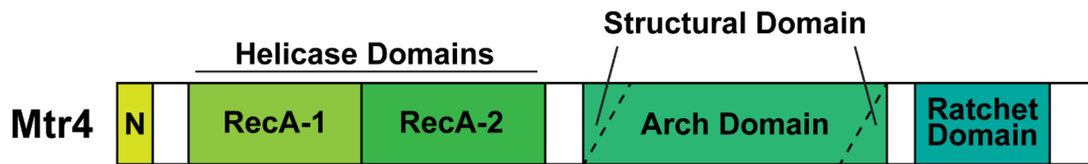


Figure 4-3: Domain structure of Mtr4. The N-terminal domain (N) is where Mtr4 interacts with Rrp47-Rrp6. RecA-1 and RecA-2 helicase domains are required for helicase and ATPase activities of Mtr4. The arch domain is required for RNA binding, processing, and degradation. The ratchet domain contributes to the unwinding rate of RNAs.

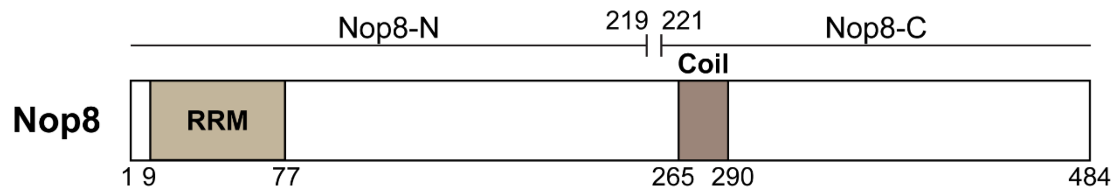


Figure 4-4: Domain structure of Nop8. The N-terminal RRM domain is an RNA recognition motif required for RNA binding. The coiled-coil domain facilitates protein-protein interactions between Nop8 and Dbp6, Nip7, and/or Rrp6.

Chapter 5

Materials and Methods:

5.1 Materials and Methods – Chapter 2

Saccharomyces cerevisiae strains and plasmids

Wildtype strain ACY233 (W303) and integrated mutant *nab2-C437S* strain ACY1026 (W303 background) were used in this study. Plasmids used in this study are listed in Table S1. Plasmid cloning was performed using the NEB Hifi assembly kit per manufacturer's instructions. A 2μ *URA3* vector was employed for overexpression of high copy suppressor genes. Myc-tagged plasmids were constructed using empty 2μ *URA3* vector pAC3007 containing 2x-Myc and *ADH1* terminator sequences as the template. For domain analysis, various deletions and amino acid substitutions in Nrd1 and Ski7 were created based on structural information available [120, 128].

Saccharomyces cerevisiae transformations and growth assays

Yeast cells were grown overnight at 30°C to saturation in minimal media lacking uracil. Cell concentrations were measured and normalized to OD_{600}= 0.4 and serially diluted in 10-fold dilutions. Cells were mixed and spotted onto minimal media plates lacking uracil and incubated at 18°C, 25°C, 30°C, and 37°C. Cells were also serially diluted and spotted on YPD plates for controls. Plates incubated at 25°C, 30°C, and 37°C were imaged after one and two days. Plates incubated at 25°C were also imaged after three days of growth. The plates grown at 18°C were imaged after three, four, and five days.

Total RNA isolation

RNA was isolated from 2 mL liquid cultures grown overnight at 30°C to saturation, diluted into 10 mL cultures at OD_{600} = 0.4, and then grown at 25°C for 4 hours. Cultures were then spun down at 3,000 x g and resuspended in 1 mL TRIzol (Invitrogen). Cells were disrupted with glass beads using the Biospec Mini Bead Beater 16 Cell Disrupter at 25°C for 1 min, placed back on ice, and then disrupted again for 1 min. 100 µL of 1-bromo-3-chloropropane was added to each sample, followed by 15 sec on the vortex and a 2 min incubation at 25°C. Samples were then centrifuged at 16,000 x g at 4°C for 8 min. The upper layer of each sample was transferred to a fresh microcentrifuge tube, precipitated with 500 µL isopropanol, and then vortexed for 10 sec. RNA was centrifuged at 16,000 x g at 4°C for 8 min, and the pellet was washed with 1 mL of 75% ethanol. Each sample was centrifuged again at 16,000 x g at 4°C for 5 min and then air-dried. RNA was resuspended in 50 µL diethylpyrocarbonate [DEPC (Sigma)]-treated water to either be DNase-treated or frozen at -80°C. 2.5 µg of RNA from each sample was added to 2.5 µL amplification-grade DNase-I (Invitrogen), 2.5 µL DNase-I Reaction Buffer, and DEPC-treated water up to a volume of 25 µL. The reactions were incubated at 25°C for 15 min, followed by inactivation of DNase-I by the addition of 2.5 µL of EDTA 25 µM solution. Reactions were incubated at 65°C for 10 min and either frozen at -80°C or converted to cDNA.

cDNA preparation and quantitative RT-PCR

1 µg DNase-treated RNA from each sample was reverse-transcribed to cDNA using M-MLV Reverse Transcriptase (Invitrogen) according to the manufacturer’s protocol. 1 µg

DNase-treated RNA from each sample was simultaneously PCR amplified without M-MLV Reverse Transcriptase to be used as a control. For quantitative PCR, 10 ng of each sample (technical triplicates of 3 independent biological replicates) was amplified using various primers (0.5 μM; Table S2) and SYBR Green PCR master mix (QIAGEN). Reactions were run for 44 cycles on a StepOnePlus Real-Time PCR machine (Applied Biosystems) with an annealing temp of 55°C. Statistical means were calculated and compared using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Mean RNA levels from experimental samples were normalized to mean RNA levels from wildtype samples, and normalized means were graphed as relative fold change of experimental samples to wildtype with error bars representing standard deviation.

RNA-sequencing and computational analysis

2 μg total RNA from each sample were analyzed for quality (Bioanalyzer), depleted of rRNA (Illumina RiboZero Gold kit), and prepared into reverse-stranded libraries (Roche KAPA kit). RNA samples were sequenced on the NextSeq PE75 High-Output Flow Cell platform. Biological duplicates were prepared and processed for all conditions. Paired-end raw reads were concatenated using Galaxy [175]. Reads were aligned to *Saccharomyces cerevisiae* S288C genome assembly R64.1.1 (SGD) using STAR [176], and counted using featureCounts [177]. Gene annotations were downloaded from SGD, and CUTs and SUTs were also annotated [2]. Raw read counts were rlog normalized for principal component analysis (PCA) and hierarchical clustering. Differential expression analysis was performed using the DESeq2 package (R V 3.6.2, [178]). Differential expression results were calculated based on gene expression of experimental samples

compared to gene expression of WT samples or gene expression of nab2 mutant samples. Gene ontology was performed using Gene Set Enrichment Analysis (GSEA 4.1.0) [179, 180]. Samples were run using a pre-ranked gene list generated from the DESeq2 output. Adjusted p-values of genes were normalized using log base 10 and ranked from highest to lowest. We ran 1000 permutations and excluded gene sets with >500 or <15 genes per GSEA default.

Data visualization

R (v3.6.2) was used to generate figures from normalized read counts or reads from differential expression analysis. Figures were built with the following R packages: ggplot2 [181], gplots [182], and RColorBrewer [183].

5.2 Materials and Methods – Chapter 3

DNA preparation

Yeast cultures were grown overnight to saturation in 10 mL YEPD. Samples were poured into 15 mL conical tubes and spun down for 3 minutes at 3000 rpm. The supernatant was dumped, and cultures were resuspended in 500 µL distilled water. Cultures were then transferred to microfuge tubes and spun at 3000 rpm for 1 minute. The supernatant was dumped and pellets were resuspended in the residual liquid. 200 µL detergent lysis buffer (2% Triton x 100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl pH8, 1 mM EDTA), 200 µL phenol chloroform, and approximately 300 mg acid-washed glass beads were added to each sample tube. Samples were vortexed for 3-4 minutes. 200 µL Tris-EDTA (TE) buffer solution (pH 8) and was added to each tube, and samples were spun for 5 minutes at 13,000 rpm. The top liquid layer of each tube was transferred to new tubes. 1 mL of cold ethanol (100%) was added to each and mixed by inverting 4 times. Samples were spun at 13,000 rpm for 2 minutes at 4°C. Supernatant was dumped, and samples were resuspended in 400 µL TE (pH 8) and 3 µL RNase. Samples were incubated for 15 to 20 minutes at 37°C, and then 10 µL of ammonium acetate (4 M) and 1 mL of cold ethanol (100%) were added to each. Samples were mixed by inverting 4 times. Samples were spun at 13,000 rpm for 2 minutes at 4°C, supernatant was dumped, and pellets were air-dried at room temperature. Pellets were resuspended in 50 µL TE (pH 8) and stored at -20°C. DNA was digested for Southern blotting using XhoI restriction enzyme.

Southern blotting

Samples were run on 0.7% agarose gels and stained with Ethidium bromide [3 uL per 100 mL of Tris-Borate-EDTA buffer (TBE)]. Gels were washed with 0.25 M hydrogen chloride (HCl) for 15 minutes twice and then washed twice for 15 minutes with 0.5 M sodium hydroxide (NaOH) and 1.5 M sodium chloride (NaCl). Gels were then washed once for 30 minutes with 0.5 M Tris (pH 7.4) and 1.5 M NaCl. Gels were rinsed with water between each wash. Blots were transferred overnight and then crosslinked. Blots were prehybridized in 15 mL of Church buffer [0.5 M sodium phosphate buffer, 1 mM EDTA, 7% SDS, and 1% bovine serum albumin (BSA)] in the hybridization oven for several hours. Blots were hybridized overnight at 55°C with approximately 10 million counts of radioactive TG1-3 probe. Blots were then rinsed with wash buffer (0.1x SSC and 0.1% SDS) and incubated for 15 minutes in hybridization oven three times. Blots were exposed to phosphoimager for a minimum of 3 hours.

Chapter 6:

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