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Characterization of mutations that rescue cytotoxicity of pre-mRNA splicing inhibition

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Characterization of mutations that rescue cytotoxicity of pre-mRNA splicing inhibition

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctorate of Philosophy in Molecular Biology

by

Samantha Robin Edwards

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ABSTRACT OF THE DISSERTATION

Characterization of mutations that rescue cytotoxicity

of pre-mRNA splicing inhibition

by

Samantha Robin Edwards

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2021

Professor Tracy L. Johnson, Chair

Pre-mRNA splicing is an essential step in expression of eukaryotic genes. In *Saccharomyces cerevisiae,* pre-mRNA splicing plays an important role in regulating proliferation and the cellular response to changes in nutrient availability. To study how defects in splicing affect cellular viability we performed a genetic screen to identify mutations that enhance growth of the temperature sensitive strain *prp2*-1, which contains a point mutation in the gene encoding the essential DEAH-box ATPase, Prp2. We identified intragenic mutations within *PRP2* that enhance growth of the temperature sensitive allele *prp2-1.* A majority of these intragenic suppressor mutations were clustered around the ATP binding site of Prp2. The intragenic *prp2* mutations identified by the screen also rescued the splicing and rRNA processing defects observed in *prp2-1*. This work provides insight into the mechanism by which the *prp2-1*, a commonly used allele to study the function of Prp2 and pre-mRNA splicing, affects RNA processing and cellular viability.

Our genetic screen also identified mutations in the stress granule component, Pbp1, which improved the growth of *prp2-1,* but did not enhance splicing. We find that in addition to decreased production of functional mRNAs, inhibition of splicing in yeast leads to sequestration of Pbp1 in the nucleus during nutrient deprivation and prevention of Pbp1 from localizing to starvation-induced cytoplasmic stress granules. We find that removal of the putative RNA binding domain of Pbp1 prevents sequestration of Pbp1 in the nucleus and restores Pbp1 cytoplasmic condensate formation during nutrient deprivation. Together this work provides insight into the ways defects in pre-mRNA splicing affect cellular function and has implications for the study of human diseases that are impacted by mutations in splicing factors, such as various cancers and neurodegenerative diseases.

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2021

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Chapter 1: INTRODUCTION

Eukaryotic genes contain sequences that encode for a functional gene product, exons, that are interrupted by non-coding sequences, introns. Introns are removed through the process of pre-mRNA splicing. Pre-mRNA splicing consists of two transesterification reactions catalyzed by the spliceosome, a complex of over one hundred proteins and five snRNAs. As pre-mRNAs are transcribed, short sequences within the intron, splice sites, are recognized by components of the spliceosome and the large complex assembles on the RNA[1]. Following assembly of the complex, the spliceosome undergoes ATP-dependent structural rearrangements to form a catalytically competent structure that facilitates the first step of splicing[2]. The first step involves nucleophilic attack by an adenosine within the branch point sequence to the 5' splice site. This first reaction results in a free first exon that remains bound by the spliceosome and an intron lariat-second exon intermediate. Following the first step, the spliceosome undergoes a series of rearrangements to facilitate the second step of splicing, ligation of the two exons, and release of the intron lariat. Finally, the spliceosome is disassembled and recycled for the next splicing reaction[3]. The dynamic rearrangements of the spliceosome structure are catalyzed by DExD/H box ATPases[2]. These tweaks to the spliceosome structure throughout the pre-mRNA splicing reaction function to ensure proper recognition of splice sites and fidelity in the timing of splicing catalysis[4].

Pre-mRNA splicing shapes the proteome, in part, through regulated inclusion or exclusion of exonic sequences in the final mRNA product. In addition, retention of intronic sequences can induce degradation of the pre-mRNA by the nuclear exosome or, if the intron contains a pretermination codon and is exported into the cytoplasm, the RNA may be degraded through nonsense-mediated decay[5,6]. Intron retention is the major form of alternative splicing in fungi and plants[7,8]. In mammals, retention of specific introns can result in the detention of polyadenylated mRNAs within the nucleus until a signal induces their posttranscriptional splicing[9].

In yeast, pre-mRNA splicing and intron retention play a vital role in regulating the cellular response to nutrient availability and ribosome biogenesis. A majority of intron containing genes encode ribosomal proteins[10]. During periods of high nutrient availability, there is increased demand for splicing machinery due to transcriptional activation of highly expressed ribosomal protein transcripts. Recent studies have demonstrated that splicing components are limited under these conditions and highly transcribed ribosomal protein transcripts outcompete more lowly expressed transcripts for the splicing machinery[11–13]. When nutrients are scarce transcription of ribosomal protein transcripts is repressed, resulting in increased splicing efficiency of other intron-containing transcripts. Recently, two studies described a phenomenon whereby intronic sequences confer a competitive advantage for survival during nutrient scarcity[14,15]. As yeast cells deplete nutrients from media they accumulate intronic sequences. Parenteau *et al.* observed an accumulation of unspliced RNAs that are stabilized by structures within the 5' end of the pre-mRNA when yeast are grown to stationary phase[14]. Morgan *et al.* demonstrated that particular linear excised introns escape degradation during nutrient deplete growth conditions or upon inhibition of the nutrient signaling complex, TORC1[15]. Both groups suggested that splicing factors bind to these stabilized intronic sequences, resulting in down regulation of ribosome biogenesis and translation through the sequestration of splicing factors from of pre-mRNAs that encode ribosomal proteins when nutrients are scarce. Future studies are necessary to determine which factors prevent degradation of stabilized intronic sequences and where these introns are located within the cell. In addition, we have studied the role of intron retention in regulating glycolysis in response to changes in environmental resources through the generation of alternative protein isoforms of the transcription factor Gcr1. As cells enter stationary phase, the splicing efficiency of *GCR1* decreases and an alternative translation start site within the intron is utilized to generate a Gcr1 protein isoform with an alternative N-terminus[16]. The Gcr1 isoforms form homo- and heterodimers that bind to DNA and regulate the expression of glycolytic genes in response to nutrient availability (Figure 1.1)[16]. These studies

are interesting examples of a functional role for intronic sequences in the regulation of gene expression, which does not involve targeting pre-mRNAs for degradation.

In this thesis we discuss mutations that improve the viability of *prp2-1,* a strain with a deleterious point mutation in the gene encoding Prp2, an essential DEAH-box ATPase required for the first step of splicing. We performed a genetic screen using temperature sensitive strains containing point mutations in splicing factors to identify factors that regulate pre-mRNA splicing. We identified several intragenic mutations that suppress the slow growth phenotype and splicing defects of *prp2-1*, which are described in Chapter 2. In addition, we identified suppressor mutations within the regulator of nutrient response, Pbp1, that rescue the slow growth phenotype of *prp2-1*, but do not rescue the splicing defect of mutant splicing factor strains. Proposed mechanisms by which mutations in Pbp1 allow cells to bypass defects in splicing and improve viability are described in Chapter 3.

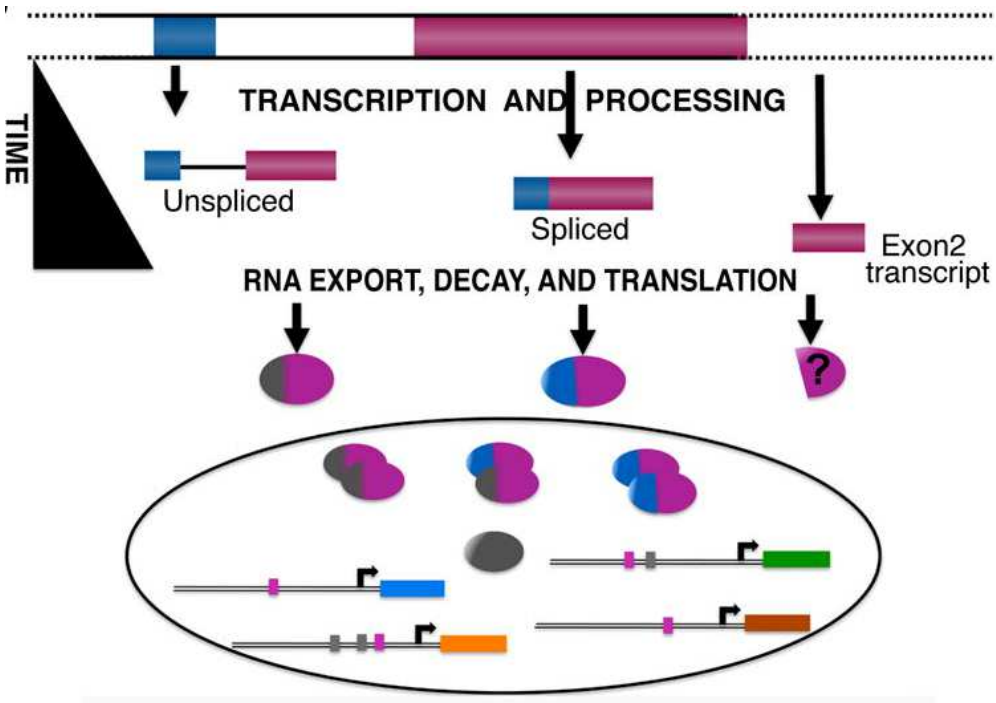


Figure 1.1 Retention if the *GCR1* intron results in translation of an alternative Gcr1 protein isoform during nutrient depleted conditions. Gcr1 isoforms derived from the spliced and unspliced transcripts can form homo- and heterodimers that regulate the transcription of glycolytic genes. Figure from Hossain et al., 2016[16].

Chapter 2: Analysis of intragenic mutations that rescue pre-mRNA splicing activity of a mutant DEAH-box ATPase

During pre-mRNA splicing, the spliceosome undergoes multiple ATP-dependent structural rearrangements that ensure fidelity of the pre-mRNA splicing reaction. These rearrangements are catalyzed by DExD/H box ATPases[2]. The DEAH box ATPase, Prp2, is essential for pre-mRNA splicing in yeast and cells lacking this gene are inviable. Prp2 catalyzes critical structural rearrangements that create the catalytically competent spliceosome complex required for the first transesterification reaction, in particular removal of the SF3b complex from the branch site[18,19]. Interaction between Prp2 the G-patch protein Spp2 is critical for coupling Prp2-catalyzed ATP-hydrolysis with rearrangement of the spliceosome structure[19–22].

We performed a genetic screen using a transposon-based yeast genomic disruption library and the temperature sensitive *prp2-1* strain to identify factors that regulate pre-mRNA splicing in yeast[23] (Figure 2.1A). The *prp2-1* strain contains a point mutation, G360D, that confers a temperature sensitive phenotype and decreased splicing efficiency at elevated temperature[24]. The genome of strains that suppressed the *prp2-1* temperature sensitive phenotype was sequenced to determine the location of the transposon disruption in the suppressors and insertion sites were confirmed by PCR using a primers specific to the transposon and the flanking endogenous DNA. We identified several suppressors with transposon insertions in the rDNA repeats located on chromosome XII. The high copy number of the rDNA gene loci may have lead to a bias for insertions in this region in the transposon library. Upon further inspection of the genomes sequenced from suppressors containing insertions within the rDNA locus, we found that many of the rDNA insertion strains also had intragenic mutations in the *PRP2* gene. The intragenic *prp2* mutations were sufficient to suppress the *prp2-1* temperature sensitivity independent of the rDNA transposon insertion. The most frequently identified intragenic suppressor (6/12 strains) was a missense mutation that changed the G360D mutation of the *prp2-1* strain to G360Y. In addition, we identified six other unique intragenic suppressors: V225I, M245T, T248S, L256I, L576P, and L607M. These

mutations were mapped on the structure of Prp2 (Figure 2.1B). The V225I, M245T, T248S, L256I mutations cluster near the ATP binding site of Prp2, suggesting that these mutations may alter ATP hydrolysis by Prp2. The L607M mutation is near the original G360D mutation and may aid in stabilizing the structure of Prp2 at elevated temperature. The G260Y mutation was the best suppressor of the temperature sensitive phenotype and these strains grow similar to WT at 34°C (Figure 2.1C). The intragenic mutations V225I and L607M modestly suppress the temperature sensitivity and we focused on these strains for analyzing of suppression of the RNA processing phenotypes of *prp2*-1.

To determine whether the intragenic suppressors Prp2^{G360D,V225I} and Prp2^{G360D,L607M} suppress the splicing defect associated with the temperature sensitivity of *prp2-1* we analyzed splicing of four intron-containing genes using reverse transcriptase PCR. After shifting the *prp2-1* strain to the nonpermissive temperature for two hours, these cells accumulate unspliced RNA (Figure 2.2A). The accumulation of unspliced transcripts is partially reduced in the suppressors and there is an increase in the relative abundance of the spliced RNA (Figure 2.2B). Thus, these suppressors partially restore the splicing activity of Prp2.

Pre-mRNA splicing is integral to production of ribosomes in yeast. Unlike metazoan genomes in which a majority of genes contain introns, only 5% of budding yeast genes contain introns. Importantly, the largest class of intron containing genes in yeast are ribosomal proteins and about two thirds of all ribosomal protein genes contain introns[10]. As a consequence, many splicing factors were initially identified as genes that affect the maturation of RPG transcripts[25]. An early study that identified *PRP2* as an essential gene required for splicing of ribosomal protein gene transcripts also determined that the *prp2-1* strain accumulates 35S pre-rRNA and displays a decrease in the mature 18S and 25S rRNAs at the nonpermissive temperature[26]. To determine whether the intragenic suppressors also rescue the rRNA processing defects of the *prp2-1* strain we analyzed rRNA processing in the suppressor strains by Northern blot (Figure 2.3B). WT, *prp2-1,* Prp2^{G360D,V225I} and Prp2^{G360D,L607M} strains were grown at the permissive

temperature (25°C) to log phase and then split into two cultures, one culture was incubated at 25°C and the other was incubated at the nonpermissive temperature (34°C) for 2 hours. Since the suppressors grow slightly slower than wild type strains we predicted that suppression of *prp2-1* is not complete, therefore, we also performed this experiment with *prp2-1,* Prp2^{G360D,V225I} and Prp2^{G360D,L607M} strains that were transformed with a plasmid encoding the wild type *PRP2* gene. This allows us to determine whether the addition of functional Prp2 would fully rescue the rRNA processing defect. The *prp2-1* strain displays an rRNA processing defect resulting in accumulation of the 35S precursor and a decrease in the 20S precursor at 25°C and 34°C (Figure 2.3B, lanes 2 and 9). The *prp2-1* strain also displays a large accumulation of 21S rRNA, an aberrant rRNA species that is typically observed in strains with mutations in the endonucleases and processing factors required for 20S rRNA production. This suggests that pre-mRNA splicing may be essential for 20S rRNA processing, perhaps through regulating the production of ribosomal proteins that assemble on the 20S rRNA precursors while the rRNA is processed. The rRNA processing defect of *prp2-1* is rescued by expression of a wild type copy of the *PRP2* gene from a plasmid (Figure 2.3B lanes 3 and 10). The intragenic suppressors Prp2^{G360D,V225I} and Prp2^{G360D,L607M} also rescue the *prp2-1* processing defect and display levels of 35S that are similar to WT with and without *PRP2*.

Discussion

This work provides insight into how the G360D point mutation of the *prp2-1* strain, which is commonly used in genetic screens to study Prp2 function and pre-mRNA splicing, results in a temperature sensitive phenotype. Future studies are needed to determine how the intragenic mutations affect stability of the Prp2 protein. These mutations may also enhance interactions between Prp2 and components of the spliceosome, such as its cofactor, Spp2. This could be tested through co-immunoprecipitation experiments to detect interaction between Prp2 and Spp2 or analysis of Prp2 recruitment to spliceosomal complexes using *in vitro* pre-mRNA

splicing assays. In addition, analysis of ATP binding and hydrolysis using the mutated Prp2 proteins may provide insight into the affects of the mutations that cluster around the ATP binding site on Prp2's catalytic function.

The observation that 20S rRNA processing is particularly affected by the *prp2-1* mutation may suggest that splicing of particular transcripts is critical for this step. Assembly of several ribosomal proteins on the rRNAs occurs during processing, and may affect the folding and maturation of the rRNAs[27]. Therefore, regulation of the production of particular ribosomal proteins through pre-mRNA splicing may influence 20S rRNA processing. Expression of ribosomal protein genes that assemble on the 20S rRNA lacking their intronic sequences in *prp2-1* may determine whether pre-mRNA splicing affects rRNA processing by regulating the production of particular ribosomal proteins. Another possibility is that pre-mRNA splicing may be required for the production of snoRNAs that are critical for processing of the 20S rRNA, as some snoRNA genes are nestled within the introns of other genes.

Methods

Yeast culture and growth assays

For dilution series, cultures were grown at 25°C to log phase (0.6-0.8OD) and then diluted to 0.1OD. The 0.1OD culture was then serially diluted ten-fold and spotted on YPD (1% yeast extract, 2% peptone, 2% glucose, 2% agar) plates and incubated at the indicated temperature. For RT-PCR and Nothern blot analysis, cultures were initially grown at 25°C until OD 0.3 and then split into two cultures. One culture was incubated with shaking at 25°C and the other was incubated at 30°C for two hours. Five milliliters of cells was collected by centrifugation and flash frozen for RNA isolation.

Mapping of suppressor mutation to the Prp2 structure

The residues mutated in the intragenic suppressors were mapped on the structure of Prp2 from *C. thermophilium* (PDB:6faa) using Pymol.

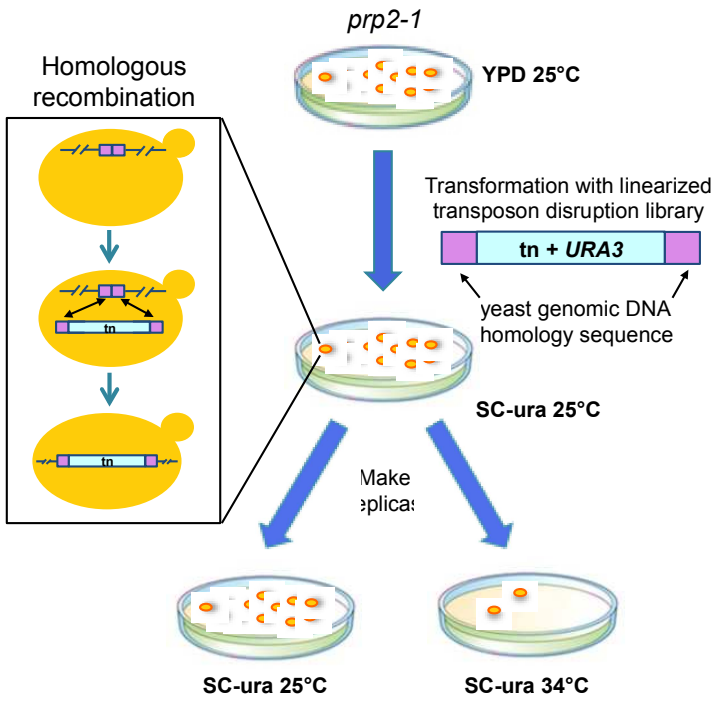
RT-PCR and Northern Blots

RNA was isolated from flash frozen cell pellets using standard phenol chloroform extraction. For RT-PCR 20ug of RNA was treated with DNAse I (Roche) and 1ug of DNAse-treated RNA was used for cDNA synthesis using the Maxima first strand cDNA synthesis kit (Thermofisher #K1641). cDNA was diluted 1:4 and 1ul was used for PCR using the primers listed in Table 2.1. Northern blots were performed as in Hossain et al[16] using radio-labeled oligo probes that anneal to the 20S and 27S rRNA precursors between the D and A2 cleavage sites and the A2 and A3 cleavage sites. Probe sequences are listed in Table 2.1.

Table 2.1 Primers used in Chapter 2

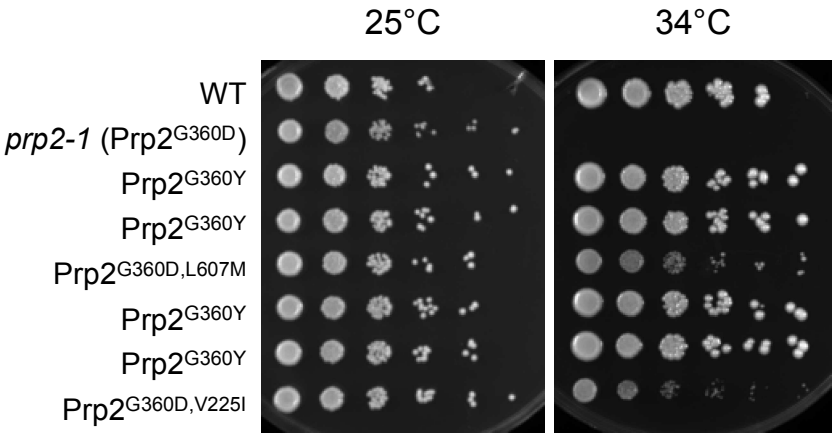
Primer name	**Sequence**
A2-A3 oligo probe	GATTGCTCGAATGCCCAAAGAAAAAGTTGC
D-A2 oligo probe	CAAAAGCACAGAAATCTCTCACCGTTTGGAATAG
SCR1 oligo probe	CAAAGATCGATTTATTATAGCCGGGACACTTCAG
HPC2 F	CCTCCACGA CCATATTCAA ACGATTGG
HPC2 R	GGAACCAGAAATTATAATGGGAGACGG
SUS1 F	GAAGTAA CAATTCTGGC CTTCACTC
SUS1 R	GGTGCATTTTCGTATCCTTCATTGTG
RPL14A F	CAAGGCTTCT AACTGGAGAT TAGTCG
RPL14A R	CAATCTTCTTAGCCCAAGATGAAGC
MUD1 F	CGGCC TCATCAAACC TAAAGAAACC
MUD1 R	GAAACCGGTCTGCTTCTTCTTGAG

A



Selection of suppressors

B



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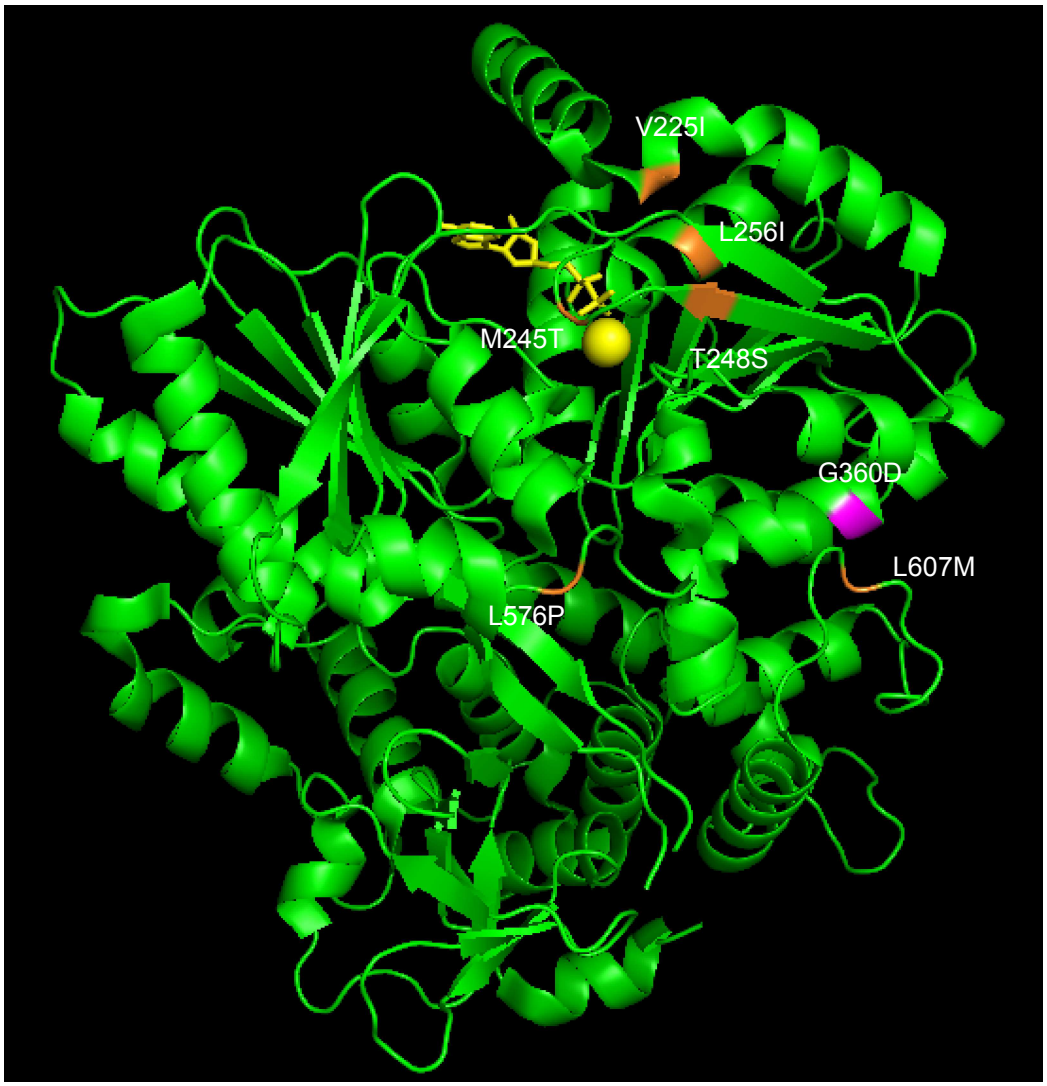

Figure 2.1 Intragenic mutations that suppress *prp2-1* (A) Schematic of the transposon-based mutagenesis screen to identify suppressors of *prp2-1.* (B) Dilution series of select *prp2-1* suppressors. Cultures were serially diluted tenfold and spotted on YPD. Plates were incubated for two days at permissive temperature (25°C) and nonpermissive temperature (34°C). (C) *prp2-1* G360D (pink) mutation and the intragenic suppressor mutations (orange) mapped on the structure of Prp2 from *Chaetomium thermophilum* (PDB:6faa).

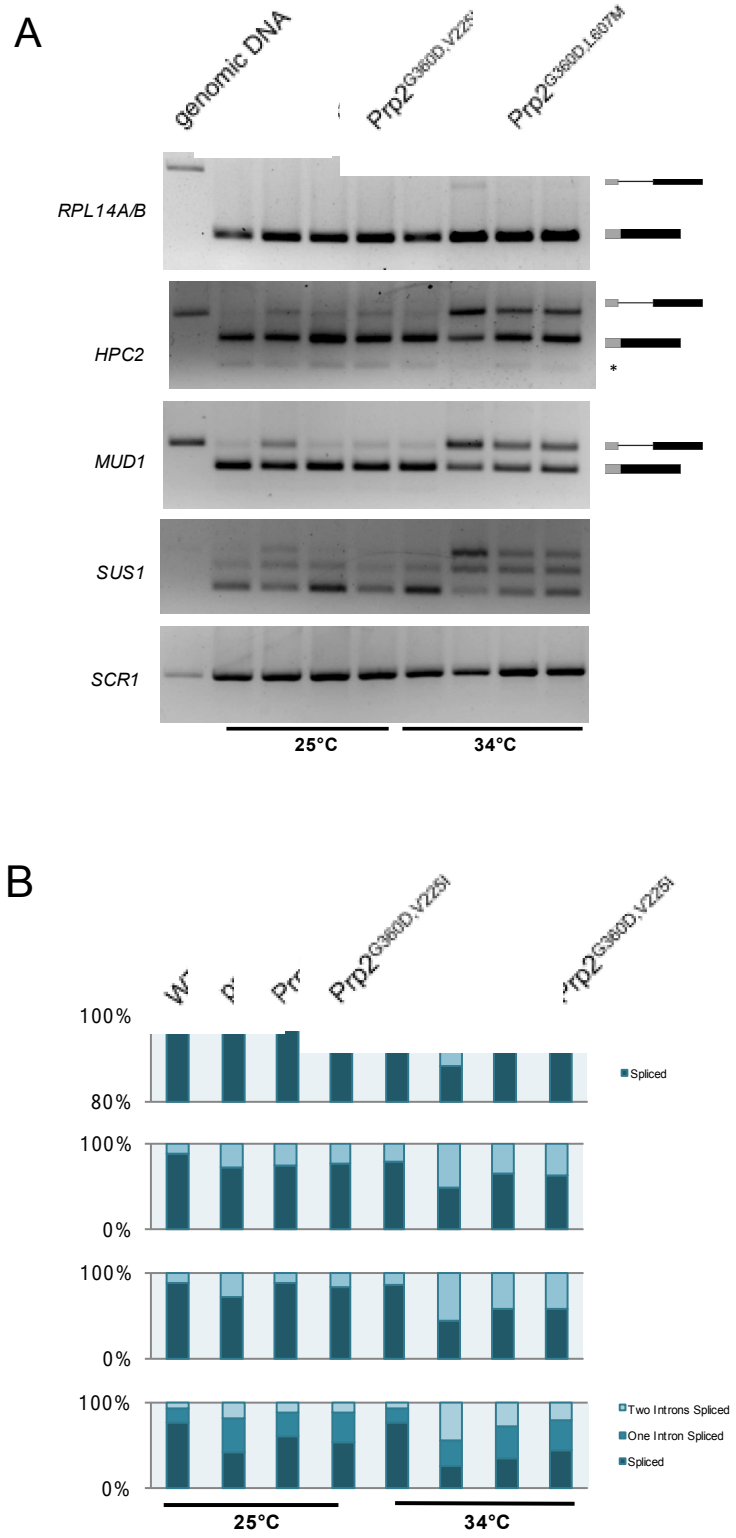

Figure 2.2 Intragenic mutations rescue defects of *prp2-1* (A) RT-PCR analysis of select intron-containing genes. (B) Quantification of the splicing analysis in (A).

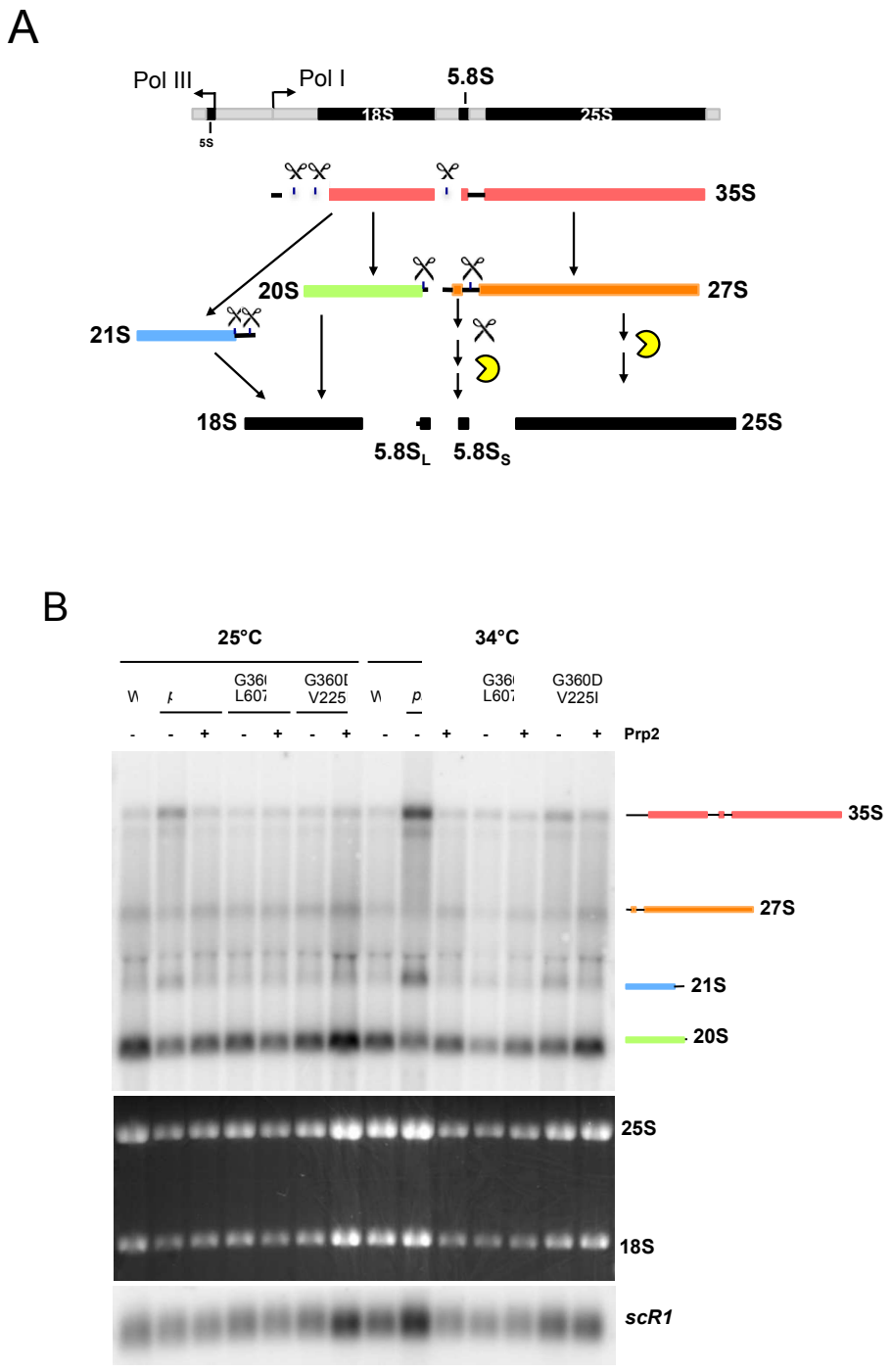

Figure 2.3 Intragenic mutations rescue rRNA processing defects of *prp2-1* (A) Schematic of rRNA processing in yeast. (B) Northern blot using probes that anneal to that anneal to the 20S and 27S precursors. *scR1* is a loading control.

Chapter 3: Mutations in the stress granule protein Pbp1 promote survival during splicing inhibition

Introduction

Eukaryotic genomes contain noncoding sequences, introns, which interrupt the protein-coding sequences of genes, or exons. Removal of intronic sequences is catalyzed by the spliceosome. The spliceosome is composed of over one hundred proteins and five snRNAs. Once splice site sequences within the nascent RNA are synthesized, they are immediately recognized by the splicing machinery[1]. The spliceosome assembles to form a catalytic center for two-step removal of the intronic sequence and subsequent ligation of the flanking exons[28]. Mutations in splicing factors contribute to a number of diseases including various cancers, retinitis pigmentosa, and neurodegenerative diseases including amyotrophic lateral sclerosis and spinocerebellar muscular atrophy[29].

Intronic sequences offer an abundance of regulatory potential. Alternative splicing, the selective removal of introns and ligation of different combinations of exons, can lead to the expression of multiple protein isoforms from one gene locus. Retention of intronic sequences frequently leads to degradation of the RNA through nonsense mediated decay or by the nuclear exosome[30]. The yeast *Saccharomyces cerevisiae* is a model system that has revealed a number of fascinating examples of regulated intron retention in response to environmental and stress conditions. For example, intron retention in yeast can generate alternative protein isoforms by readthrough translation and regulate the use of alternative translation start sites within the intronic sequence[12,16]. Regulated intron accumulation in response to nutrient depletion facilitates proper cellular response and survival during stationary phase. Two independent studies revealed that stabilization of intronic sequences during stationary phase promotes survival and downregulates splicing of ribosomal protein genes in a manner dependent on TORC1 nutrient signaling[14,15,17].

Yeast genetic screens that identify suppressors of splicing factor mutations have been utilized to probe the network of interactions in the spliceosome. We also hypothesized that a genetic screen might reveal gene products that mediate the cellular effects of intron

accumulation. To this end, we performed a screen to identify gene disruptions that enhance growth in cells with splicing factor mutations and identified a mutation within *PBP1*, the yeast ortholog of Ataxin-2, which rescued the slow growth phenotypes displayed by splicing factor mutants. Pbp1 has been extensively studied as a component of stress granules and, along with its eukaryotic orthologs, has been implicated in a number of cellular functions including inhibition of nutrient signaling[31], lipid droplet formation[,32], endoplasmic reticulum dynamics[33], regulation of polyA tail length[34,35], and targeting cell division factors to the spindle midbody[36]**.** Pbp1 and its orthologs in other eukaryotic species share a conserved domain architecture comprised of an Lsm (Like-Sm) domain, an Lsm associated domain (LsmAD), and prion like domains with low amino acid sequence complexity that facilitate the aggregation of Pbp1/Ataxin-2 *in vivo* and *in vitro*[37].

Lsm domains are RNA binding domains that often form multimeric structures with other Lsm proteins, for example the heteroheptameric complex comprised of Lsm2-8 involved in pre-mRNA splicing and the Lsm1-7 complex involved in cytoplasmic degradation of pre-mRNAs in P bodies[38–40]. Pbp1 interacts the Lsm protein Lsm12 and was not found to directly bind to other Lsm proteins found in the spliceosome or in P bodies[41]. Ataxin-2 can bind RNA directly, with a preference for U-rich RNA elements in 3'UTRs of a subset of mRNAs[42]. Binding to these elements is independent of Ataxin-2's interaction with the polyA binding protein, PABPC1[42]. Axatin-2 bound RNAs were enriched for transcripts of genes that regulate metabolic processes, RNA splicing, mRNA polyadenylation and 3'end processing[42]. Additionally, proteomics data from yeast and mice demonstrate that deletion of *PBP1* results in reduced expression of metabolic enzymes; however, the mechanism by which deletion of *PBP1* contributes to decreased expression of metabolic proteins is unclear[43,44]. Pbp1/Ataxin-2 has been proposed to regulate mRNA stability[42]. Pbp1 was initially identified as a protein that interacts with the polyA binding protein Pab1 and simultaneous deletion of *PBP1* and *PAB1* results in extended polyA

tails[45]. Pbp1 also appears to act as a negative regulator of polyA endonuclease, which may contribute to Pbp1's effect on RNA stability[46].

Pbp1/Ataxin-2 regulates nutrient sensing through inhibition of TORC1. During nutrient deprivation and acute stress, Pbp1 condenses into cytoplasmic stress granules along with its binding partners Pab1, Lsm12, and Pbp4[47]. Stress granules are formed through inhibition of translation initiation, followed by aggregation and binding of RNA binding proteins to translationally inhibited RNAs[48]. RNAs contained within stress granules do not appear to be enriched for a particular process, but are biased toward longer mRNAs[49]. During heat shock and glucose deprivation Pbp1 is phosphorylated by Psk1 and sequesters the TORC1 nutrient signaling complex into stress granules[50–52]. Inhibition of TORC1 is conserved in higher eukaryotes. Ataxin-2 knockout cells display increased phosphorylation of downstream TORC1 signaling targets following serum starvation and loss of Ataxin-2 leads to obesity in mice and increased cell size and lipid storage in c. elegans[31,32].

Ataxin-2 has largely been studied in the context of neurodegenerative disease. Intermediate polyQ expansions (27-33 glutamines) in the N-terminus of Ataxin-2 are associated with increased susceptibility to and early onset of amyotrophic lateral sclerosis (ALS)[53] and large polyQ expansions (>33 glutamines) are the genetic cause of spinocerebellar ataxia 2 (SCA2)[54]. PolyQ expansions of Ataxin-2 induce aberrant localization and aggregation of the splicing factor TDP-43 in cell models of ALS and neurons derived from ALS patients[55]. Deletion of *PBP1/ATXN2* relieves toxicity of aggregates caused by transgenic expression of TDP-43 in yeast and mice[53,56,57]. Mutations in splicing factors, including TDP-43, and RNA binding proteins that regulate RNA processing and transport have been linked to ALS[58]. Altered splicing patterns have been observed in ALS patient derived neurons and TDP-43 transgenic mouse models[59–61], but the contribution of these altered splicing events to disease pathology are unclear. Moreover, the relationship between the combined RNA processing defects and Ataxin-2 mutations in ALS has not been characterized.

Here we demonstrate that the removal of the putative RNA binding domains and/or the C-terminal low complexity sequence of Pbp1, enhances the growth of cells harboring mutations in splicing factors that function throughout the splicing cycle. Surprisingly, *pbp1* mutations do not increase splicing efficiency in these strains. Moreover, deletion of the region that facilitates Pbp1-mediated TORC1 inhibition is detrimental to the growth of splicing factor mutants. To understand how *pbp1* mutations suppress cytotoxicity of splicing inhibition, we focus on the effects of *pbp1* mutations on the splicing factor mutant *prp2-1.* This strain contains a point mutation, G360D, in the gene encoding the DEAH box ATPase Prp2 that prevents formation of a catalytically competent spliceosome[19,24]. Inhibition of pre-mRNA splicing in *prp2-1* cells results in retention of the transcripts of intron-containing genes in the nucleus[62]. Splicing inhibition in the *prp2-1* mutant leads to sequestration of Pbp1 in the nucleus and prevents formation of starvation-induced cytoplasmic Pbp1 condensates. Inhibition of splicing also delays downregulation of TORC1 nutrient signaling. Surprisingly, this delay is not rescued by mutations in *PBP1* that enhance growth in *prp2-1*. Mutations in *PBP1* that enhance growth in *prp2-1* do, however, rescue formation of cytoplasmic Pbp1 condensates. Together these data provide a picture of how perturbations in splicing cause cellular toxicity, not only through the reduction in functional mRNA products, but also through mislocalization of Pbp1 under nutrient scarce conditions.

Results

Deletion of the putative RNA binding domains and low complexity region of Pbp1 enhances growth in a splicing factor mutant

To identify suppressors of splicing mutants we performed a disruption mutagenesis screen using temperature sensitive splicing mutants and identified a gene disruption within the 3' end of *PBP1* that enhances growth of splicing factor mutants[23]. The Pbp1 gene disruption is

predicted to truncate the C-terminus of Pbp1 within the low complexity domain (LCD), a region of low amino acid sequence complexity that facilitates homotypic interaction and Pbp1 condensate formation in yeast[50,52]. To determine which regions of Pbp1 affect growth in splicing factor mutants we generated a series of deletions to remove functional regions of the protein. The N-terminus of Pbp1 contains two putative RNA binding domains, the Like Sm (Lsm) domain and Lsm-associated domain (LsmAD). The C-terminus of Pbp1 contains a region that facilitates binding to Kog1 (KB), a component of the TORC1 signaling complex, and the LCD[51,52] (Figure 3.1A). Deletion of each domain alone or both of the RNA binding domains did not affect growth when spotted on YPD (Figure 3.2). To analyze the effect of *pbp1* mutations on growth in cells that accumulate unspliced RNA we introduced these deletions into the *prp2-1* strain, which contains the point mutation G360D in the gene encoding the DEAH box ATPase Prp2[24]. Prp2 catalyzes rearrangements of spliceosomal proteins that result in the formation of the catalytically competent spliceosome[19]. The *prp2-1* strain displays a slow growth phenotype at 30ºC (Figure 3.1B). Removal of the low complexity domain conferred suppression of the *prp2-1* growth defect, consistent with this region being identified in our initial screen. Deletion of the putative RNA binding domains, *pbp1*∆*Lsm* or *pbp1*∆*LsmAD,* individually or in combination (*pbp1*∆*LsmLsmAD)* displayed a stronger rescue than removal of the LCD. Additionally, deletion of the entire *PBP1* gene improves growth of *prp2-1.* We observe a similar pattern of growth when these strains are grown in liquid cultures, particularly as cells deplete nutrients, around the post-diauxic shift (Figure 3.1C).

During stress such as nutrient starvation and heat shock, Pbp1 inhibits the TORC1 signaling cascade by binding the TORC1 component Kog1 and sequestering the complex into stress granules[51,52]. Unlike the other *pbp1* mutants, we observed that deletion of the region that binds Kog1 does not improve growth in *prp2-1,* but in fact inhibits growth of *prp2-1* cells. Since deletion of the entire gene enhances growth, while deletion of just the Kog1 interacting region

inhibits growth, we hypothesize that the *pbp1ΔKB* mutation generates a dominant negative Pbp1 isoform in *prp2-1.*

To determine the generality of *pbp1* mutant effects on splicing factor mutations, we analyzed the *pbp1* mutations in combination with deletions of factors that act throughout the splicing cycle. Deletion of the Lsm domain also improves growth in cells deleted of the splicing factors Lea1, Msl1, and Dbr1, (U2 snRNP factors and the debranching enzyme, respectively) while deletion of the Kog1 interacting region inhibits growth in these strains (Figure 3.3). This suggests that mutations within the putative RNA binding domains of Pbp1 generally suppress growth defects associated with increased abundance of unspliced RNA and removal of the Kog1 binding region is detrimental in splicing factor mutants.

PBP1 mutations that improve the growth of *prp2-1* do not increase splicing efficiency

When grown at elevated temperature *prp2-1* cells display a global splicing defect[63]. To determine whether *pbp1* mutations that enhance growth improve splicing efficiency in *prp2-1* cells we assayed splicing of intron containing transcripts that have varying expression levels and functions, but have all been shown to be sensitive to Prp2 activity. *SUS1* is a lowly expressed gene with two introns and contains a nonconsensus 5' splice site within the first intron. SEC27 contains a single intron with consensus splicing signals and the protein product of *SEC27* is involved in the secretory pathway. *RPL16A* is a highly abundant transcript that encodes a ribosomal protein gene. RT-PCR utilizing primers that flank the introns was used to analyze splicing of these transcripts. The *prp2-1* strain accumulates unspliced RNA for each transcript assayed. However, mutations that enhance growth, e.g. deletion of *PBP1* or the Lsm domain, do not rescue the splicing defect of *prp2-1* (Figure 3.4), indicating a lack of correlation between improved cell viability and improved splicing. In addition, *prp2-1 pbpΔKB,* which displays a decreased growth rate than *prp2-1*, is not correlated with a decrease in splicing efficiency. In fact, *prp2-1 pbp1ΔKB* displays a similar splicing phenotype to the mutations that

enhance growth, despite the strong growth defect. These results suggest that the mutations in Pbp1 do not affect survival through a mechanism that is directly related to their effects on splicing outcomes.

Splicing inhibition prevents Pbp1 cytoplasmic condensate formation during nutrient deprivation and glucose starvation

We observe that the greatest difference in growth between the *prp2-1* strain and the *pbp1* mutations that enhance growth occurs after 24 hours in liquid culture, as the cells begin to enter stationary phase (Figure 3.1C). Stationary phase is a period of decreased proliferation that is triggered by inhibition of nutrient signaling pathways due to depletion of nutrients, including glucose, from growth media[64]. An important feature of the yeast response to nutrient deprivation and stress is the formation of stress granules, cytoplasmic condensates composed of translationally inhibited RNAs and RNA binding proteins[65]. Pbp1 is a component of these cytoplasmic condensates that form during glucose depletion[66]. Therefore, we next considered the possibility that Pbp1's effects on formation of RNA-protein condensates during stationary phase might contribute to the deleterious growth effects in *prp2-1*. During log phase growth, we observe Pbp1 localization in the nucleus and the cytoplasm in wild type cells, consistent with data from previously published cellular fractionation assays (Figure 3.5A)[35]. Wild type cells accumulate Pbp1 condensates after one day of growth and most cells have Pbp1 condensates after 2 days of growth (Figure 3.5A). In addition to formation of stress granules, the transition to stationary phase in wild type cells induces accumulation of stable intronic sequences, which was previously demonstrated to help cells adapt to decreased nutrient availability by downregulation ribosome biogenesis[14,15]. We observe accumulation of intronic sequences in wild type cells around 16 hours of growth, just before wild type cells enter stationary phase (Figure 3.5B and Figure 3.1C). In contrast, the splicing factor mutant strain *prp2-1* accumulates unspliced RNA during early log phase (6 hours of growth) (Figure 3.5B). After one day of

growth, Pbp1 localization within and around the nucleus increases and after two days of growth, Pbp1 localization in the nucleus decreases in some *prp2-1* cells, but cytoplasmic condensates are not observed (Figure 3.5A). Pbp1 localization dynamics in response to nutrient changes is disrupted in *prp2-1* cells.

Fluorescent *in situ* hybridization experiments demonstrated that *prp2-1* accumulates transcripts of intron-containing genes in the nucleus[62]**.** We hypothesize that inhibition of Pbp1 condensate formation in *prp2-1* may be caused by the early accumulation unspliced RNA in the nucleus. This increased local concentration of nuclear unspliced RNA may induce condensation of Pbp1 within this compartment and prevent its translocation during nutrient deprivation. Consistent with this, *in vitro* experiments that examined conditions that promote protein condensation demonstrated that pre-assembled RNA condensates are capable of recruiting Pbp1[67]**.** Additionally, ALS-associated interactions between Ataxin-2 and TDP-43 are RNA dependent[56]**.** These results suggest that accumulation of unspliced RNAs in the nuclei of *prp2-1* cells may facilitate aberrant interactions between Pbp1 and RNA and/or RNA binding proteins.

We next tested whether mutation of the Pbp1 Lsm domain, a putative RNA binding domain, affects Pbp1 localization during nutrient deprivation. During long-term growth, *pbp1*Δ*Lsm* cells display Pbp1 puncta localized near the nucleus after two days (Figure 3.6). Similarly, smaller Pbp1 puncta proximal to the nucleus are observed in *prp2-1* cells when the Lsm domain is removed from Pbp1. In contrast, *pbp1*Δ*KB* cells display small condensates and more diffuse cytoplasmic Pbp1 compared to wild type or *pbp1*Δ*Lsm* cells. A similar distribution of Pbp1ΔKB-GFP is observed in the *prp2-1 pbp1*Δ*KB* cells. The correlation of enhanced growth, Pbp1 puncta formation, and decreased Pbp1 nuclear localization observed in *prp2-1pbp1*Δ*Lsm* cells suggests that the improved viability of this strain may be the result of relieving stress caused by nuclear accumulation of Pbp1 and/or prevention of Pbp1 binding to unspliced RNA in the nucleus. There may be competition between RNAs or proteins bound by Pbp1 that function in nuclear processes under normal growth conditions and RNA and protein interactions that

regulate the cellular response to stress via Pbp1 condensate formation in the cytoplasm**.** The increase of unspliced RNA in nuclei of *prp2-1* cells may tip the balance toward Pbp1 nuclear interactions despite environmental changes that would induce cytoplasmic Pbp1 functions.

We tested whether the *prp2-1* mutation also alters Pbp1 condensates that are triggered by glucose deprivation. Wild type cells that are transferred to media lacking glucose form cytoplasmic Pbp1 condensates (Figure 3.7). Formation of Pbp1 condensates is abolished during glucose deprivation in *prp2-1.* In media lacking glucose, Pbp1-GFP primarily co-localizes with the nuclear marker Htb1-mCherry in *prp2-1* cells. Deletion of the Pbp1 Lsm domain in *prp2-1* restores Pbp1 cytoplasmic condensate formation during glucose starvation. Similar to our observations after two days of growth, deletion of the Lsm domain results in fewer Pbp1 condensates compared to wild type cells and the Pbp1ΔLsm condensates are primarily located near the nucleus. This suggests that the protein or RNA components present in Pbp1 condensates formed in the absence of the Lsm domain may contain different constituents or interact with different organelles than Pbp1 condensates formed in wild type cells.

Pbp1 is predicted to form a heterodimer via its Lsm domain with Lsm12[41]. Lsm12 and Pbp1 were previously identified in a large-scale assay to identify proteins that interact with the spliceosome[68] and may function together in the nucleus during co-transcriptional RNA processing events. To determine if disruption of predicted nuclear spliceosomal interactions can suppress the *prp2-1* mutant, *LSM12* was deleted in combination with the *prp2-1* mutation. Deletion of *LSM12* enhances growth in a *prp2-1* strain, though to a lesser extent than *pbp1*Δ (Figure 3.8). This suggests that disruption of Pbp1 nuclear interactions contribute to suppression of *prp2-1.* In addition, if the Lsm12-Pbp1 interaction helps facilitate Pbp1 binding to RNA, suppression of *prp2-1* by deletion of *LSM12* may support the model that reduction of Pbp1 binding to RNA enhances viability in *prp2-1* cells.

Inhibition of TORC1 signaling during long term growth is delayed in *prp2-1*, but is not rescued by mutations in *PBP1*

Depletion of nutrients from liquid media during long-term growth in culture leads to inhibition of TORC1 signaling, which inhibits ribosome biogenesis and induces autophagy[69,70]. Under these conditions in wild type cells, Pbp1 inhibits TORC1 signaling by binding to the TORC1 component Kog1 and sequestering Kog1 and Tor1 kinase into stress granules[51,52]. The *prp2-1* strain does not form Pbp1 cytoplasmic condensates during nutrient deplete conditions, but removal of the Pbp1 Lsm domain can restore cytoplasmic condensate formation in *prp2-1* cells. Therefore, we considered the possibility that TORC1 inhibition is disrupted in *prp2-1* cells and may be rescued by deletion of the Pbp1 Lsm domain. To assess the effects of the *prp2-1* mutation on TORC1 signaling, we assayed phosphorylation of Rps6. Rps6 is phosphorylated in a TORC1-dependent manner during exponential growth and is a useful readout for TORC1 activity and inhibition[71,72]. Rps6 dephosphorylation is delayed in *prp2-1* cells (Figure 3.9). The *prp2-1 pbp1∆Lsm* strain shows a minimal reduction in Rps6 phosphorylation and a similar effect was observed when the Kog1 binding region was removed. Therefore, enhanced inhibition of TORC1 signaling is not likely the cause of the increased viability of the *prp2-1 pbp1∆Lsm* strain.

Discussion

Removal the putative RNA binding domains and, to a lesser extent, the region that facilitates condensation of Pbp1 improve growth of splicing factor mutant strains. Deletion of *PBP1* may mitigate the toxic effect of unspliced RNA accumulation in the nucleus because its absence would prevent Pbp1 binding to unspliced RNA and/or formation of aberrant nuclear Pbp1 condensates. Interestingly, knockout of the mammalian ortholog of Pbp1, Ataxin-2, reduces nuclear TDP-43 inclusions in a transgenic mouse model of ALS[73]. Interaction between Ataxin-2 and TDP-43 is indirect and RNA-dependent[53]. In yeast, the accumulation of unspliced RNA in nuclei of *prp2-1* cells may result in aberrant, nuclear RNA-protein condensates via

recruitment of RNA binding proteins, including Pbp1. Additional experiments analyzing the aggregation of splicing factors under these conditions may provide useful insight into this possibility. One interesting candidate is Nam8, the yeast ortholog of Tia1, a splicing factor that assembles into stress granules and has been linked to ALS[74,75].

Wild type yeast cells accumulate unspliced RNAs and stabilize excised introns as a mechanism to downregulate ribosomal protein production when environmental resources are low[14,15]. During this period, Pbp1 condenses into cytoplasmic stress granules. However, when cells accumulate unspliced RNA during log phase, as in the *prp2-1* strain, Pbp1 does not form condensates in the cytoplasm upon entry into stationary phase. Since removal of the Lsm domain, a putative RNA binding domain, and deletion of the *PBP1* gene enhances growth of *prp2-1* we predict that disrupting Pbp1 interactions with unspliced RNA is important for suppression. The accumulation of unspliced RNA in the nuclei of *prp2-1* cells during log phase may promote Pbp1 binding to unspliced RNA causing irreversibly sequestration of Pbp1 in the nucleus. We cannot rule out the possibility that the early accumulation of unspliced RNA in *prp2-1* cells may alter signaling pathways that trigger Pbp1 translocation to the cytoplasm during nutrient depletion. Additionally, the accumulation of unspliced RNA in the nucleus of *prp2-1* cells may decrease the concentration of RNA in the cytoplasm compared to wild type cells and inhibit stress granule formation through the prevention of RNA-dependent condensation of RNA binding proteins.

Future experiments are necessary to determine whether Pbp1 binds directly to unspliced RNAs under wild type, nutrient depleted conditions and in *prp2-1* cells and whether these interactions occur in the nucleus or the cytoplasm. To determine whether unspliced RNA induces Pbp1 condensation we will perform *in vitro* condensation assays with recombinant Pbp1 and analyze whether RNA isolated from *prp2-1* cells, which is enriched with unspliced RNA, would promote condensation of Pbp1 to a greater extent than RNA isolated from wild type cells. To determine whether splicing inhibition promotes Pbp1 nuclear localization, we will induce high

copy expression of an intron-containing transcript with a branch point mutation that inhibits splicing of this transcript. If induction of this construct prevents formation of Pbp1 condensates during nutrient depletion, we could then test whether Pbp1 binds directly to this intron-containing RNA and whether binding is facilitated by the Lsm domain of Pbp1. Together these experiments will determine whether mutations in *PBP1* that suppress *prp2-1* relieve toxicity due to Pbp1 binding nuclear unspliced RNA.

Methods

Strains and plasmids

All strains used were in the BY4741 strain background. Pbp1 domain mutants in the CEN.PK background were obtained from the Tu lab[52]. Primers flanking the *PBP1* gene were used to amplify each mutant construct and transformed into *pbp1*Δ cells in the BY background. The Pbp1-GPF and Pab1-GFP strains were purchased from Open Biosystems. Htb1-mCherry tagged strains were generated by amplifying the tag from the SK1 strain from the Ünal lab[76] and transforming the Htb1-mCherry tag into *prp2-1* cells. *prp2-1 pbp1* double mutants were generated by genetic crosses.

Yeast culture conditions and growth assays

For dilution series assays cells were grown in YPD (yeast extract, peptone, 2% dextrose) to saturation and then diluted to 0.2 OD. These cultures were then grown to log phase 0.6-0.8 OD and then diluted to 0.1OD and serially diluted tenfold and spotted onto YPD agar plates. For growth curve and Western blot experiments cells were grown in SC media (complete supplement mixture (Sunrise Biosciences #1001-010, yeast nitrogen base without amino acids, 2% dextrose) starting at 0.2 OD and ODs were measured at the indicated time points. Cells for Western blot analysis were spun down and flash frozen in liquid nitrogen. Growth curves were generated in R using the drc pakage[77].

RT-PCR

Cultures were initially grown at 25°C until OD 0.3 and then split into two cultures. One culture was incubated with shaking at 25°C and the other was incubated at 32°C for two hours. Five milliliters of cells was collected by centrifugation and flash frozen. RNA was isolated from cell pellets using standard phenol chloroform extraction. 20ug of RNA was treated with DNAse I and 1ul of DNAse-treated RNA was used for cDNA synthesis using the Maxima first strand cDNA synthesis kit (Thermofisher #K1641). cDNA was diluted 1:4 and 1ul was used for PCR. Primers used are listed in Table 3.1. PCR products were run on 1.5% agarose gels.

Microscopy

Cells were grown in SC media and imaged after reaching ~0.6-0.7 OD for log phase, or after one or two days post seeding. For imaging during log phase, 0.5 OD of cells were collected by centrifugation at 3,000 x g, resuspended in 100ul of SC media, and then 4ul was spotted onto poly-L-lysine treated slides. Cells imaged one or two days post-seeding were directly spotted onto poly-L-lysine treated slides. Confocal microscopy following +/- glucose deprivation was performed using a Zeiss Lsm 710 microscope with a 63x/1.4A oil objective. Airyscan microscopy of cells grown to log phase and following two days of growth was performed on a Zeiss LSM 880 confocal microscope with Airyscan in the Broad Stem Cell Institute Research Center and Molecular, Cell and Developmental Biology microscopy core at UCLA. Images were generated using an Plan-Apochromat 63×/1.4 oil DIC M27 objective and optimal resolution and Z-stack intervals were set using Zeiss Zen Black software. Maximum intensity projections were generated in FIJI.

Western blots

Protein was isolated from 2.5 OD of flash frozen cell pellets with FA-1 lysis buffer (50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 0.1% Sodium deoxycholate, 10% glycerol) supplemented with a protease inhibitor cocktail and Phosstop phosphatase inhibitor (Roche) by 5 rounds of 1 min vortexing with acid washed beads and with 1 min rest in between vortexing at 4˚C. Protein concentrations of each sample were measured by Pierce™ BCA protein assay. Equal amounts of total protein were loaded on an 12% SDS-PAGE gels and transferred to PVDF membrane. Membranes used to detect Rps6 and p-Rps6 were blocked with 5% BSA in TBS-T (20 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% Tween-20) for one hour at room temperature and incubated overnight at 4 °C with primary antibodies diluted in TBS-T with 5% BSA (1:1,000 for rabbit anti-RPS6 (#ab40820, Abcam), 1:1,000 for rabbit anti-phospho-Ser235/Ser236-S6 (#2211, Cell Signaling Technology). Membranes were incubated for one hour at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies diluted in TBS-T with 5% BSA (1:5,000 for donkey anti-rabbit IgG Santa Cruz). Membranes used to detect PGK1 or Pbp1-FLAG were blocked with 5% milk in TBS-T for one hour at room temperature and incubated overnight at 4 °C with primary antibodies diluted in TBS-T with 5% milk (1:10,000 for mouse anti-PGK1 (Molecular Probes), 1:3,000 for mouse anti-M2 FLAG (Sigma). Membranes were incubated for one hour at room temperature with anti-mouse HRP-conjugated secondary antibodies (Roche) diluted in TBS-T with 5% milk.

Table 3.1 Primers used in this study

Primer name	**Sequence**
SUS1 F	GAAGTAA CAATTCTGGC CTTCACTC
SUS1 R	GGTGCATTTTCGTATCCTTCATTGTG
Rpl16A F	GTCTGTTGAACCAGTTGTTGTCATTG
Rpl16A R	GCTTTGTAGAAGATTCTAGATGGGGC
Sec27 F	CGGACACGATGAAGTTGGATATAAAG
Sec27 R	CTGTCAAATCATCACTGCCGG

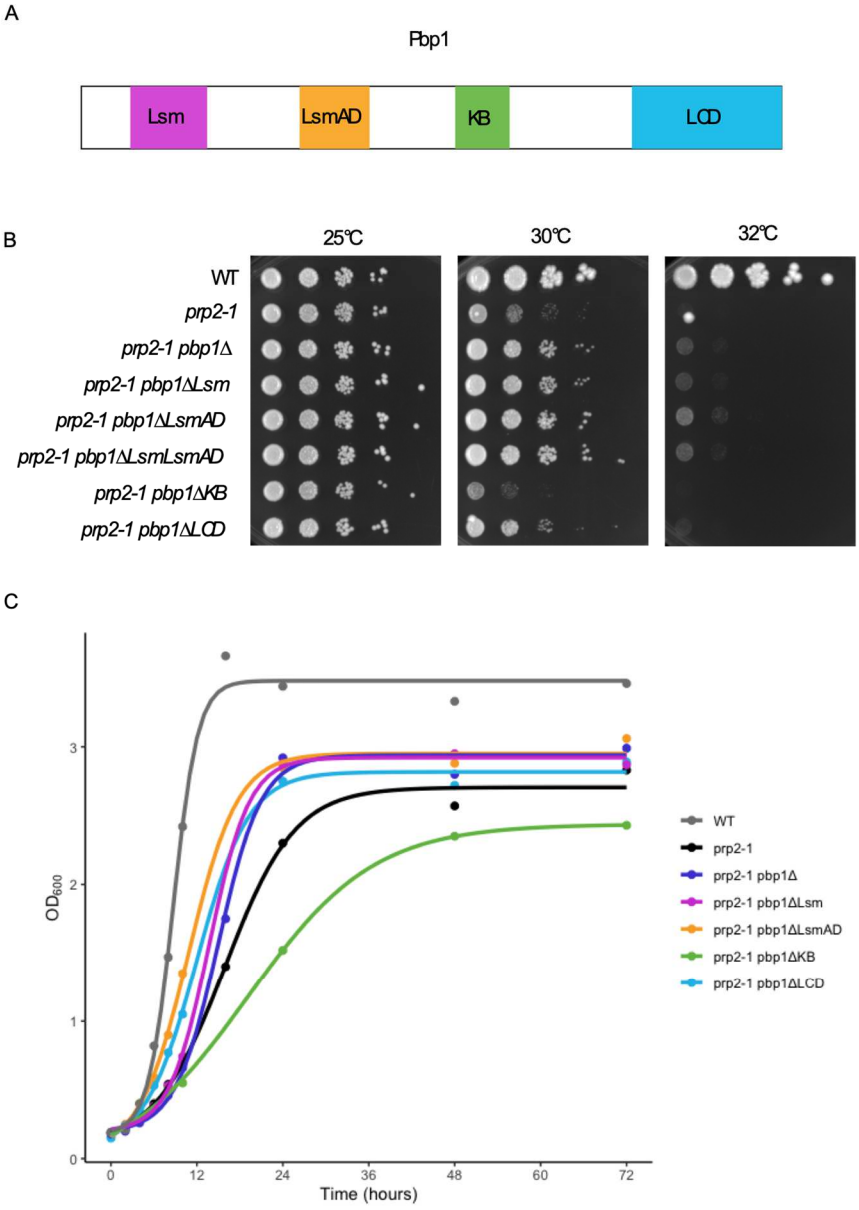

Figure 3.1 Removal of the putative RNA binding domains or low complexity domain of Pbp1 improves growth of the splicing factor mutant *prp2-1.* (A) Schematic of Pbp1 domains. (B) Yeast cultures serially diluted tenfold and spotted on YPD plates. Plates were incubated for two days at 25ºC, 30ºC, or 32ºC. (C) Growth curves generated by measuring OD_{600} readings for 72 hours show a similar growth pattern of *pbp1* mutations in *prp2-1* cells grown on agar plates.

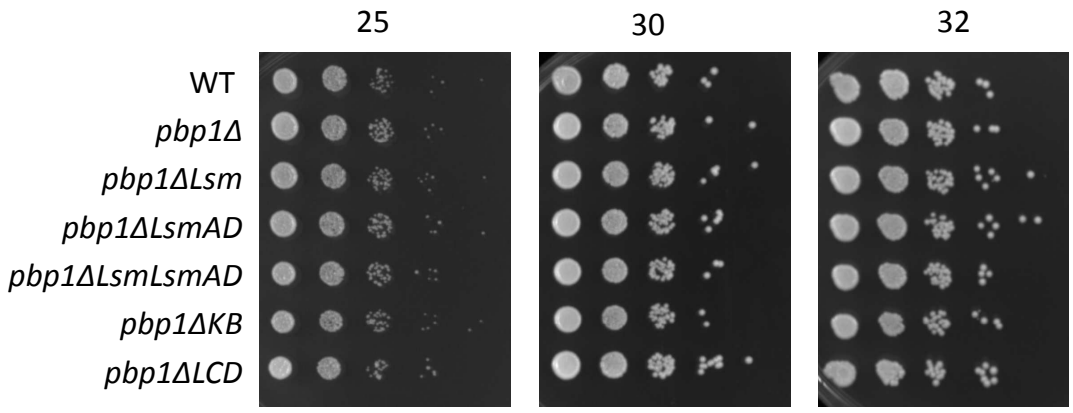

Figure 3.2 Deletion of Pbp1 domains alone has no effect on growth on YPD plates. Plates were incubated for two days at 25ºC, 30ºC, or 32ºC.

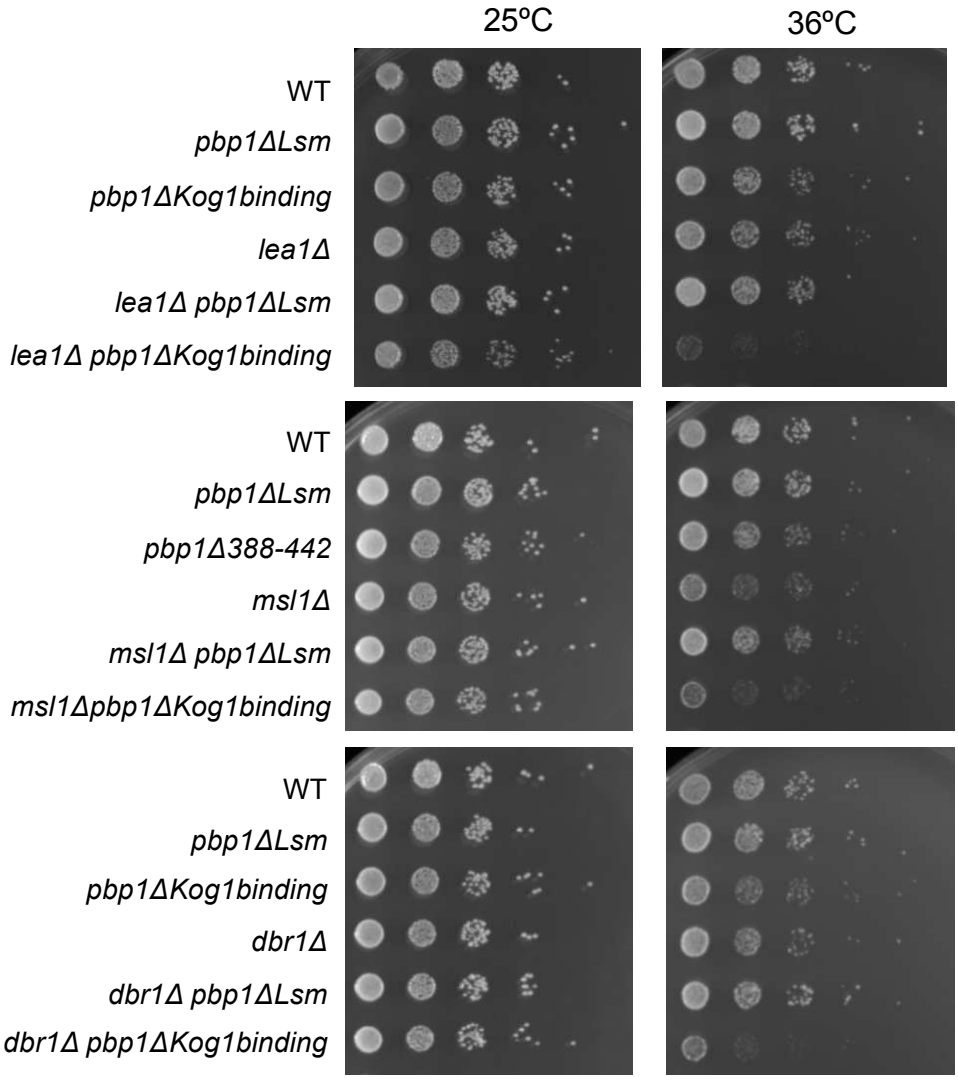

Figure 3.3 Deletion of the Pbp1 Lsm domain improves splicing factor mutant growth and deletion of the Kog1 interacting region inhibits growth. Yeast cultures serially diluted tenfold and spotted on YPD plates and incubated for two days at 30ºC or 36ºC.

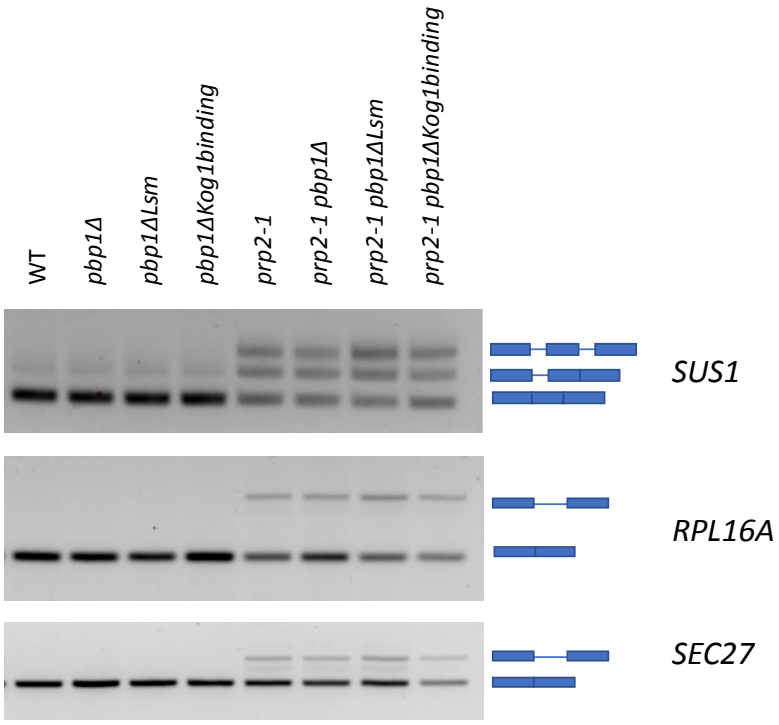

Figure 3.4 Pre-mRNA splicing defects are not rescued by mutations in *PBP1*. Primers within the first and last exon of each transcript were used to assess splicing of *SUS1, RPL16A,* and *SEC27. prp2-1* cells grown at 32ºC accumulated unspliced RNAs. Deletion of *PBP1,* the Lsm domain, or the Kog1 binding region does not enhance splicing in *prp2-1* cells.

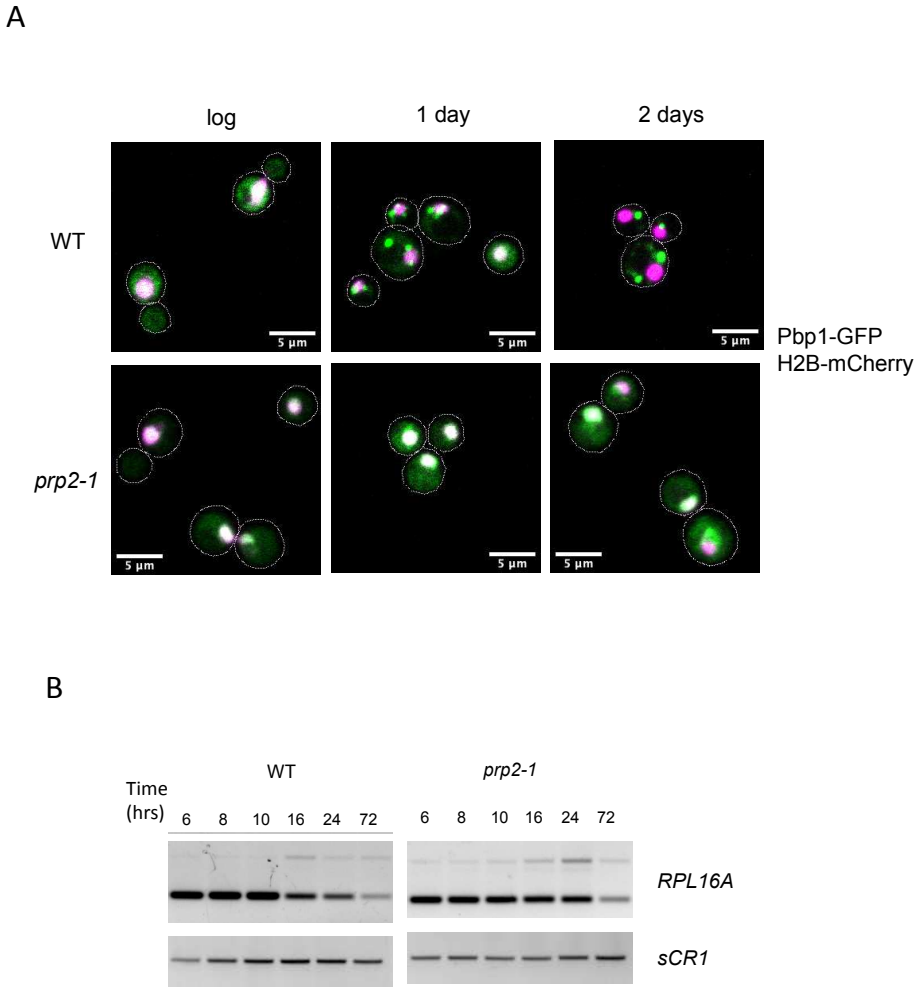

Figure 3.5 Formation of Pbp1 cytoplasmic condensates is inhibited during stationary phase in *prp2-1.* (A) WT cells begin to form cytoplasmic Pbp1 condensates after one day of growth in culture and most cells have condensates after two days. Pbp1 remains primarily in the nuclei of *prp2-1* cells throughout log and stationary phase growth. (B) The formation of Pbp1 condensates in WT cells occurs in the same time frame as increased abundance of unspliced transcripts during stationary phase growth, whereas *prp2-1* cells accumulate unspliced transcripts during exponential growth (6 hours) but do not accumulate Pbp1 condensates.

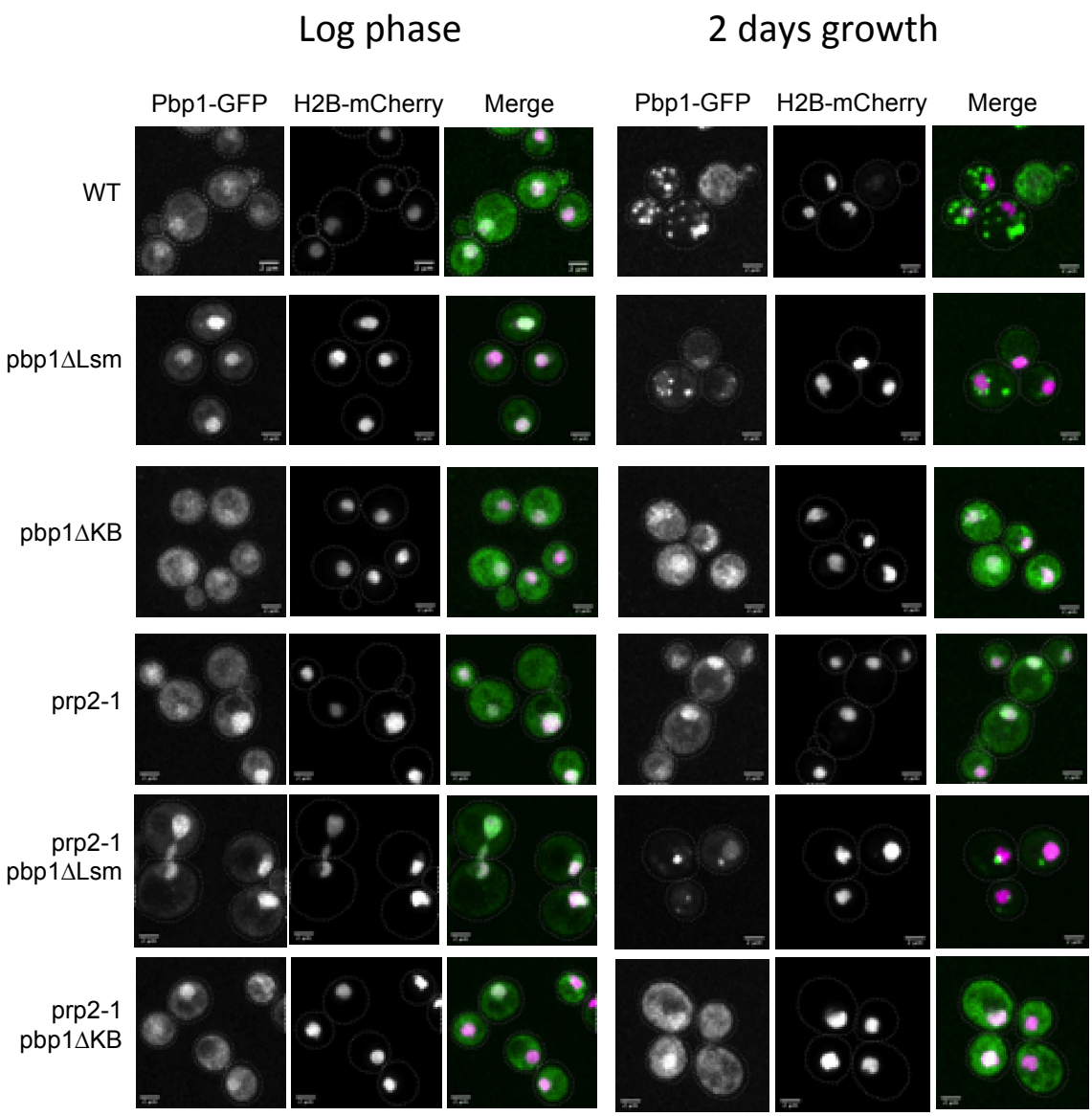

Figure 3.6 Formation of Pbp1 cytoplasmic condensates during stationary phase growth is rescued by deletion of the Lsm domain of Pbp1 in *prp2-1* cells, but deletion of the Kog1 binding domain does not result in Pbp1 condensation after two days of growth in *prp2-1.*

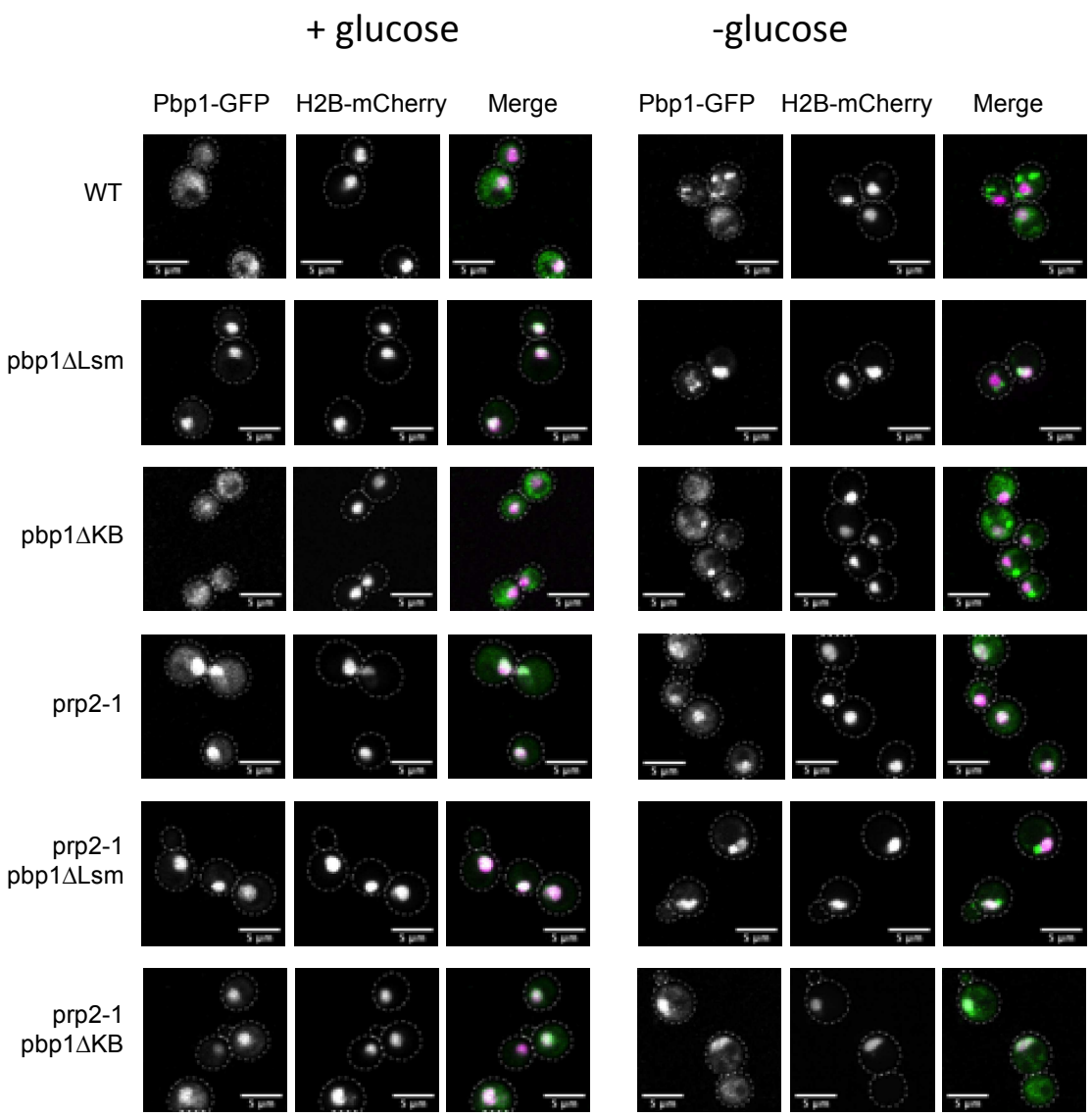

Figure 3.7 Formation of cytoplasmic Pbp1 condensates is inhibited in *prp2-1* during glucose starvation. Images of WT and *prp2-1* cells expressing Pbp1-GFP and Ht1b-mCherry during log phase growth in SC media. In WT cells Pbp1 localizes in the nucleus and diffusely in the cytoplasm. Glucose starvation induces cytoplasmic condensation of Pbp1-GFP in WT cells and Pbp1 cytoplasmic aggregation is inhibited in *prp2-1.* Deletion of the Pbp1 Lsm domain in *prp2-1* rescues formation of Pbp1 condensates during glucose starvation. Pbp1 condensate formation is not rescued by removal of the Kog1 binding domain.

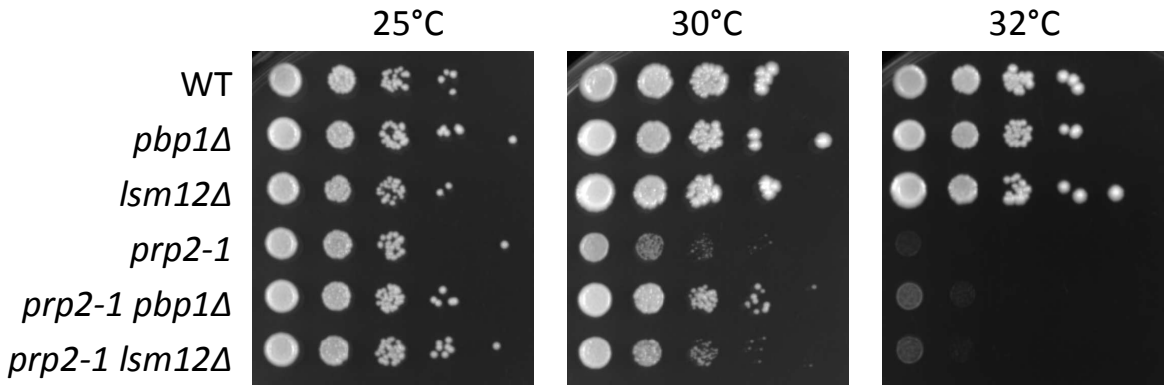

Figure 3.8 Deletion of Pbp1 binding partner Lsm12 enhances growth in *prp2-1.* Plates were incubated for two days at 25ºC, 30ºC, or 32ºC.

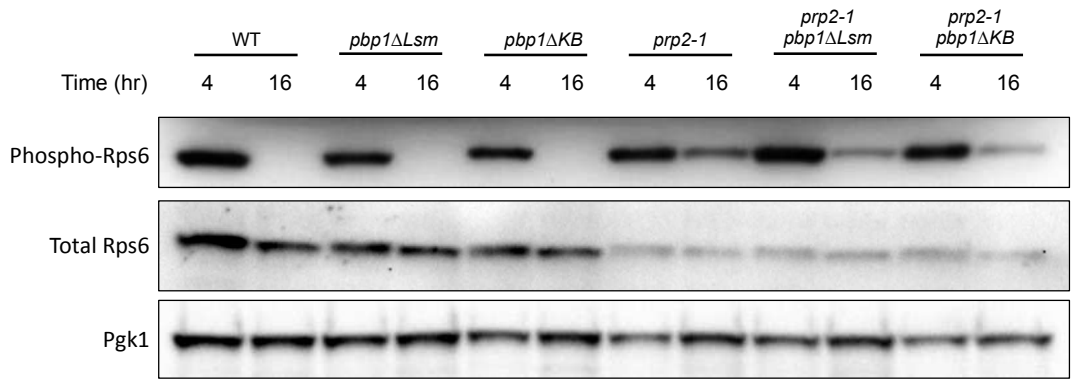

Figure 3.9 TORC1 signaling inhibition is delayed in *prp2-1* cells and this defect is not rescued by mutation of *PBP1.* Rps6 is phosphorylated during log phase growth (6 hours) in a TORC1 dependent manner in WT. *pbp1ΔLsm* and *pbp1ΔKB* show a similar trend to WT. *prp2-1* cells sustain phosphorylation of Rps6 to 16 hours of growth and the delay in TORC1 inhibition is not rescued by mutation in *PBP1.*

Chapter 4: Conclusions

Intron retention has recently been described as a mechanism to control localization of RNAs. Polyadenlyated RNAs with one to two introns retained can remain tethered to chromatin to control timed expression of particular transcripts[9]. This mechanism is particularly important for neurogenesis and neural activity[78]. A subset of polyadenylated neuronal transcripts retain select introns and are detained in the nucleus until neuronal activity induces posttranscriptional splicing, export of the mRNA into the cytoplasm, and translation[79]. Motor neurons differentiated from human induced pluripotent stem cells harboring ALS-causing mutations display increased cytoplasmic localization of unspliced RNAs[80]. The intron retained transcripts are bound by the RNA binding proteins TDP-43, SFPQ, and FUS, which display nuclear localization in wild type neurons, but form cytoplasmic foci in neurons with ALS-causing mutations[80]. Therefore, aberrant localization and accumulation of RNA containing intronic sequences in the cytoplasm may be a driver of RNA binding protein mislocalization in ALS.

We show that deletion of Pbp1 reduces toxicity associated with inhibition of splicing and retention of unspliced RNA in the nucleus. We propose a model in which Pbp1 directly or indirectly binds unspliced RNA in the nucleus during splicing inhibition in combination with nutrient deprivation and facilitates aggregation of pre-mRNA and RNA binding proteins. Removal of the putative RNA binding domain in Pbp1 or deletion of *PBP1* could prevent toxic nuclear RNA-protein aggregates from forming during splicing inhibition. This model could similarly be used to explain how mouse models of ALS that transgenically express the splicing factor TDP-43 accumulate nuclear inclusions of TDP-43, which can be resolved by knockout of the mammalian Pbp1 homolog, Ataxin-2. Ataxin-2 and TDP-43 indirectly interact in an RNA-dependent manner and Ataxin-2 displays a preference for binding polyU sequences[42,53]. Future experiments will determine whether Pbp1 binds directly to unspliced RNA in the nucleus and whether certain sequence or structural elements within introns facilitate its binding.

Wild-type cells accumulate intronic sequences following nutrient depletion during stationary phase growth[14,15]. During this period, Pbp1 forms cytoplasmic condensates. Regulation of Pbp1 translocation into the cytoplasm, potentially through posttranslational modification or inhibition of protein interactions that facilitate RNA binding, may be important to prevent Pbp1 from binding to unspliced RNA in the nucleus during stationary phase. The regulation of Pbp1 localization maybe disrupted in splicing factor mutant strains that accumulate intronic sequences during rapid growth prior to stationary phase.

We observe that cytoplasmic Pbp1 condensates do not form during stationary phase when glucose levels are low in a mutant splicing factor strain, possibly due to Pbp1 sequestration in the nucleus and the prevention of its translocation into the cytoplasm during stress. Mutations in *PBP1* that suppress this phenotype, such as removal of the Lsm domain, restore Pbp1 condensate formation during splicing inhibition. However, since deletion of the entire *PBP1* gene enhances growth to a similar extent as removal of the Lsm domain, rescue of Pbp1 condensate formation is not necessary for the enhanced viability of the splicing factor mutant strains, and is more likely driven by prevention of Pbp1 from binding RNA. Although Pbp1 and Ataxin-2 colocalize with stress granule components during acute stress, these proteins are not required for the formation of stress granules. Future microscopy experiments with fluorescently labeled stress granule components will determine whether inhibition of splicing prevents condensation of other stress granule factors or whether Pbp1 localization is specifically affected by splicing inhibition.

Together this work provides insight into mechanisms by which the accumulation of intronic sequences affects cellular viability and response to environmental changes. In addition, this work has implications for the study of Ataxin-2 and RNA binding proteins involved in pre-mRNA splicing that contribute to ALS. We find that removal of the Lsm domain of Pbp1 is a potent suppressor of splicing factor mutant growth defects and restores stress-induced formation of Pbp1 condensates, whereas removal of the region that regulates nutrient signaling

is inhibitory to growth of splicing factor mutants strains. This work may provide insight into particular regions of Ataxin-2, such as the Lsm domain, that could be targeted for therapeutics to treat ALS pathologies associated with defects in pre-mRNA processing.

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