REGULATION OF H2B MONOUBIQUITYLATION BY RNP RECYCLING PROTEIN PRP24

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ABSTRACT

Katie S. Bolling: Regulation of H2B Monoubiquitylation by RNP Recycling Protein Prp24
(Under the direction of A. Gregory Matera and Brian D. Strahl)

Post-translational modification of histone proteins and splicing of pre-mRNA transcripts are two fundamental aspects of gene regulation that are essential for eukaryotic life. Often, these processes are investigated in isolation; however, they do not occur independently. Although evidence supports cross-regulation between chromatin and RNA processing, there remains a gap in understanding of the mechanisms involved. Here, I describe a novel function for splicing protein Prp24, mutation of which alters monoubiquitylation of histone H2B (H2Bub1). Whereas the role of Prp24 in spliceosome assembly and recycling has been well studied, it has not been previously associated with a role in chromatin modification. My results indicate that a highly conserved region of Prp24 may be critical for regulating levels of H2Bub1. Investigations into the effects of U6 small nuclear (sn)RNA on this regulation are also discussed. Together, these data suggest a mechanism by which spliceosomal machinery regulates histone modification.

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LIST OF ABBREVIATIONS

DUB	Deubiquitylating Enzyme
H2Bub1	Monoubiquitylation of H2B at Lysine 120 (humans) or Lysine 123 (yeast)
PTM	Post-Translational Modification
RRM	RNA Recognition Motif
snRNA	Small Nuclear RNA
snRNP	Small Nuclear Ribonucleoproteins

Chapter I

Introduction

Expression of eukaryotic genes is a highly intricate and dynamic process involving many layers of regulation. Together, chromatin structure, cis-regulatory elements in DNA, transcription factors, RNA processing, post-translational modifications (PTMs), and numerous other factors determine how the "message" of a gene is interpreted.

One of the most significant and highly conserved steps in this process is splicing of pre-mRNA transcripts. In this process, intronic segments of RNA are removed and exons are ligated together to form the mature mRNA that will be translated into a functional protein product (Figure 1A). Splicing is carried out by the spliceosome, a complex consisting of 5 small nuclear RNAs and their associated proteins (snRNPs). U1 and U2 snRNPs bind to the 5' splice site and branch point on the intron, respectively. Afterwards, U4, U5, and U6 snRNPs, in a complex known as the tri-snRNP, enter the spliceosome. Several rearrangements take place, ending with exons being ligated together and snRNPs being released along with the intron. The snRNPs can then be recycled into pre-splicing complexes to carry out additional splicing reactions. Splicing is often considered a post-transcriptional process; however, it is widely accepted that much of splicing occurs co-transcriptionally. Given this information, it is almost certain that splicing will influence other transcriptional processes, including histone post-translational modification.

Histones are the core of the nucleosome, the basic unit of chromatin. Each nucleosome consists of 8 histones (2 copies each of H2A, H2B, H3, and H4) wrapped in approximately 150 base pairs of genomic DNA. Addition of post-translational modifications to the histone proteins can have significant impact on chromatin structure and therefore any DNA-associated process, including transcription and splicing. Although many histone modifications have been reported to influence splicing, spliceosomal influence on histone PTMs has not been reported often. However, it is not a novel concept, as splicing was shown to enhance recruitment of Setd2 to methylate H3K36 (de Almeida et al. 2011). Recent data

suggest that monoubiquitylation of H2B (H2Bub1) may be connected to splicing in budding yeast as well as in humans (Hérissant et al. 2014; Long et al. 2014). H2Bub1 (Figure 1B) is a well-conserved mark closely linked to active transcription and under tight regulation (Wozniak & Strahl 2014). Inhibiting the catalytic activity of Bre1, the E3 ubiquitin ligase responsible for H2Bub1, leads to destabilization of the protein and decreases in H2Bub1 levels. Additionally, highly active deubiquitylating enzymes (DUBs) rapidly remove the mark from H2B (Figure 1B). Whereas the direct impact of H2Bub1 on chromatin structure is debated, data support the importance of the mark in a variety of processes, including genomic stability (Northam & Trujillo 2016), transcription elongation (Pavri et al. 2006), and Notch signaling (Bray et al. 2005), among others. H2Bub1 (Briggs et al. 2002) is a prerequisite for the presence of at least two other histone marks, H3K4 and H3K79 methylation (Briggs et al. 2002; Dover et al. 2002; Sun & Allis 2002). Additionally, mutation of the human homolog of Bre1, Rnf20, as well as several DUBs, has been associated with multiple human cancers (reviewed in Cole, Clifton-Bligh, & Marsh, 2015) and deletion of Bre1 in *Drosophila* is embryonic lethal (Bray et al. 2005). Further understanding of the how this mark is regulated so closely will be of significant value in determining its impact on gene expression in development and disease. To this end, I investigated the role of splicing factors in regulating H2Bub1.

It has been suggested that Sart3, which is conserved in yeast as Prp24, may play a role in regulating H2Bub1(Long et al. 2014). Sart3/Prp24 is a snRNP recycling protein (see Figure 1A) that interacts directly with the U6 snRNA and facilitates its recycling from the post-catalytic spliceosome into the U4/U6 di-snRNP, a pre-splicing complexes that precedes tri-snRNP formation (Raghunathan 1998). The function of this protein is well conserved (Bell et al. 2002), as is ubiquitylation of H2B, supporting the idea that the influence of Prp24 on H2Bub1 may also be conserved. Additionally, the limited number of intron-containing genes in *Saccharomyces cerevisiae* (approximately 6%) make yeast an ideal organism for studying the function of splicing factors while minimizing the effects of misspliced gene products, a major caveat in higher eukaryotes. Although Prp24 is essential in budding yeast, a variety of viable point mutants affecting the various RNA-recognition motifs of Prp24 have been produced (Vidaver et al. 1999; Kwan & Brow 2005; Montemayor et al. 2014). In this study, I demonstrate the effects of one such mutant on H2Bub1, supporting a role for Prp24 in regulating post-translational modification of histones.

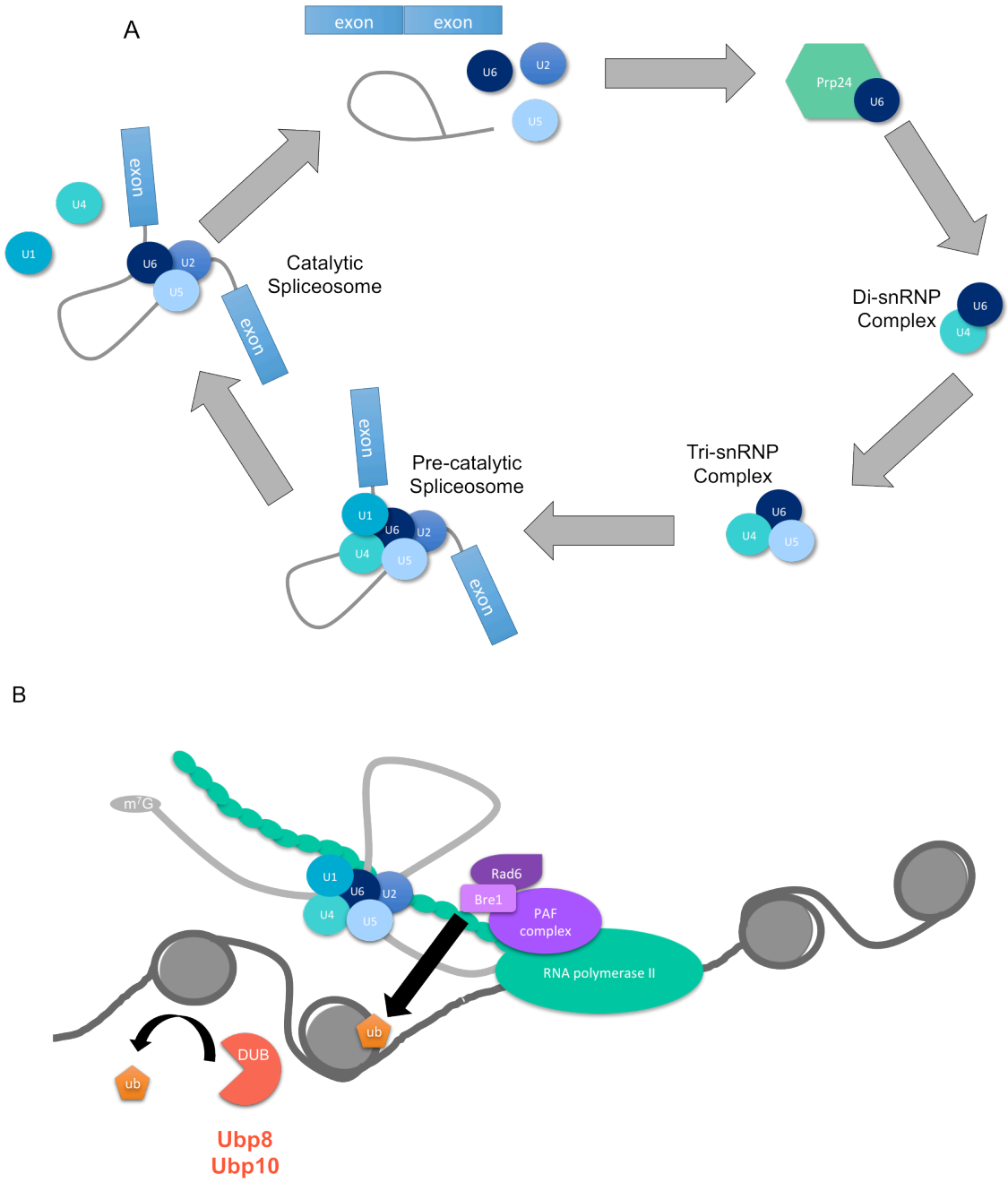

Figure 1: (A) U6 in the splicing reaction. U6 enters the spliceosome bound to U4 in the Tri-snRNP complex, interacts with U2 and the 5′ splice site of the intron to catalyze the splicing reaction, and is released along with U2 and U5. U6 is then bound by Prp24 and recycled back into the Di-snRNP complex. (B) Key players in H2B monoubiquitylation. Bre1 (E3 ubiquitin ligase) and Rad6 (E2 ubiquitin conjugating enzyme) associate with PAF complex on the C-terminal domain of RNA polymerase II during transcription elongation. Deubiquitylating enzymes Ubp8 and Ubp10 rapidly remove ubiquitylation from H2B post-transcription.

Methods

Yeast strains and plasmids

Strains and plasmids used in this study are listed in Tables 1 and 2.

Table 1: Strains

Strain	Genotype	Source
LL101	MAT**a**his3 leu2 trp1 ura3 met2 can1 ade2 lys2 prp24Δ1::ADE2 [pUN50-PRP24]	Vidaver et al. 1999
prp24RRM1sub	MAT**a**his3 leu2 trp1 ura3 met2 can1 ade2 lys2 prp24Δ1::ADE2 [pSS24-RRM1]	
LL200	MAT**a** his3 leu2 trp1 ura3 met2 can1 ade2 lys2 prp24Δ1::ADE2 snr6Δ::LEU2 [pUN50-PRP24]	Vidaver et al. 1999
prp24F87A	MAT**a** his3 leu2 trp1 ura3 met2 can1 ade2 lys2 prp24Δ1::ADE2 snr6Δ::LEU2 [pRS313-Prp24-F87A][pRS314-U6-WT]	Kwan et al. 2005
prp24S151I	MAT**a** his3 leu2 trp1 ura3 met2 can1 ade2 lys2 prp24Δ1::ADE2 snr6Δ::LEU2 [pRS313-Prp24-S151I][pRS314-U6-WT]	Montemayor et al. 2014
prp24R158S	MAT**a** his3 leu2 trp1 ura3 met2 can1 ade2 lys2 prp24Δ1::ADE2 snr6Δ::LEU2 [pRS313-Prp24-R158S][pRS314-U6-WT]	Vidaver et al. 1999
prp24M213T	MAT**a** his3 leu2 trp1 ura3 met2 can1 ade2 lys2 prp24Δ1::ADE2 snr6Δ::LEU2 [pRS313-Prp24-M213T][pRS314-U6-WT]	Montemayor et al. 2014
prp24F257I	MAT**a** his3 leu2 trp1 ura3 met2 can1 ade2 lys2 prp24Δ1::ADE2 snr6Δ::LEU2 [pRS313-Prp24-F257I][pRS314-U6-WT]	Vidaver et al. 1999
prp24S350R	MAT**a** his3 leu2 trp1 ura3 met2 can1 ade2 lys2 prp24Δ1::ADE2 snr6Δ::LEU2 [pRS313-Prp24-S350R][pRS314-U6-WT]	Montemayor et al. 2014
BY4741	MATa his3Δ1 leuΔ0 met15Δ0 ura3Δ0	Open Biosystems
ist3Δ	MATa his3Δ1 leuΔ0 met15Δ0 ura3Δ0 ist3Δ::NAT	This study
mud1Δ	MATa his3Δ1 leuΔ0 met15Δ0 ura3Δ0 mud1Δ::NAT	This study

Table 2: Plasmids

Plasmid	Features	Source
pRS315-Sus1cDNA	CEN LEU2 pSUS1-Sus1cDNA	Hossain et al. 2009
pRS424-SNR6	2μ TRP1 pSNR6-SNR6	Gift from D. Brow
FLAG-Bre1 p416 ADH	CEN URA3 pADH1-FLAG-BRE1	Wozniak and Strahl 2015

Antibodies

The following antibodies were used in this study: Ubiquityl Histone H2B Lys 120 (cell signaling cat# 5546P), Histone H2A (active motif cat# 39235), Histone H2B (active motif cat# 39237), FLAG M2 (Sigma -Aldrich cat# F3165).

Yeast Cell Extracts

A single colony was inoculated overnight to saturation and then diluted to an OD600 0.2 and grown to midlog phase. Five optical densities of cells were isolated and lysed via bead beating in SUMEB (1% SDS, 8 M urea, 10 mM MOPS, pH 6.8, 10 mM EDTA, 0.01% bromphenol blue) for 8 min. Lysates were boiled for 10 min and then isolated. Cell debris were removed via centrifugation, and the supernatant was isolated.

Western Blot Analysis

Cell lysates were loaded onto 15% SDS-PAGE gels and then transferred to PVDF membrane. Membranes were probed overnight (4 °C) with primary antibody. Western blots were visualized using HRP conjugated secondary antibodies and ECL Prime solution (GE Healthcare).

Spotting assays

Fivefold serial dilutions of saturated overnight yeast cultures were plated on YPD or SC medium. Cells were plated at a starting OD600 of 0.5 on the appropriate medium and imaged after 2–4 days of growth at 30°C or 37°C.

Results

A missense mutation in Prp24 causes severe growth defects

Because Prp24 is an essential gene, I used several point mutants to investigate the effects of reduced Prp24 function (Figure 2A), including at least one mutation in each of the 4 RRMs. With the exception of the mutations in RRM1, each point mutant alters a residue known to interact with a specific nucleotide on U6 snRNA (Montemayor et al. 2014). Mutations in RRM1 include *RRM1sub*, mutation of 3 conserved residues in the RNP-1 consensus motif of RRM1, and F87A, a mutation of an adjacent highly conserved residue. Although *RRM1sub* appears to have no significant affect, F87A causes the most severe growth defect of all mutants tested (Figure 2B). Mutations in other RRMs showed little effect on cell growth (Figure 2B).

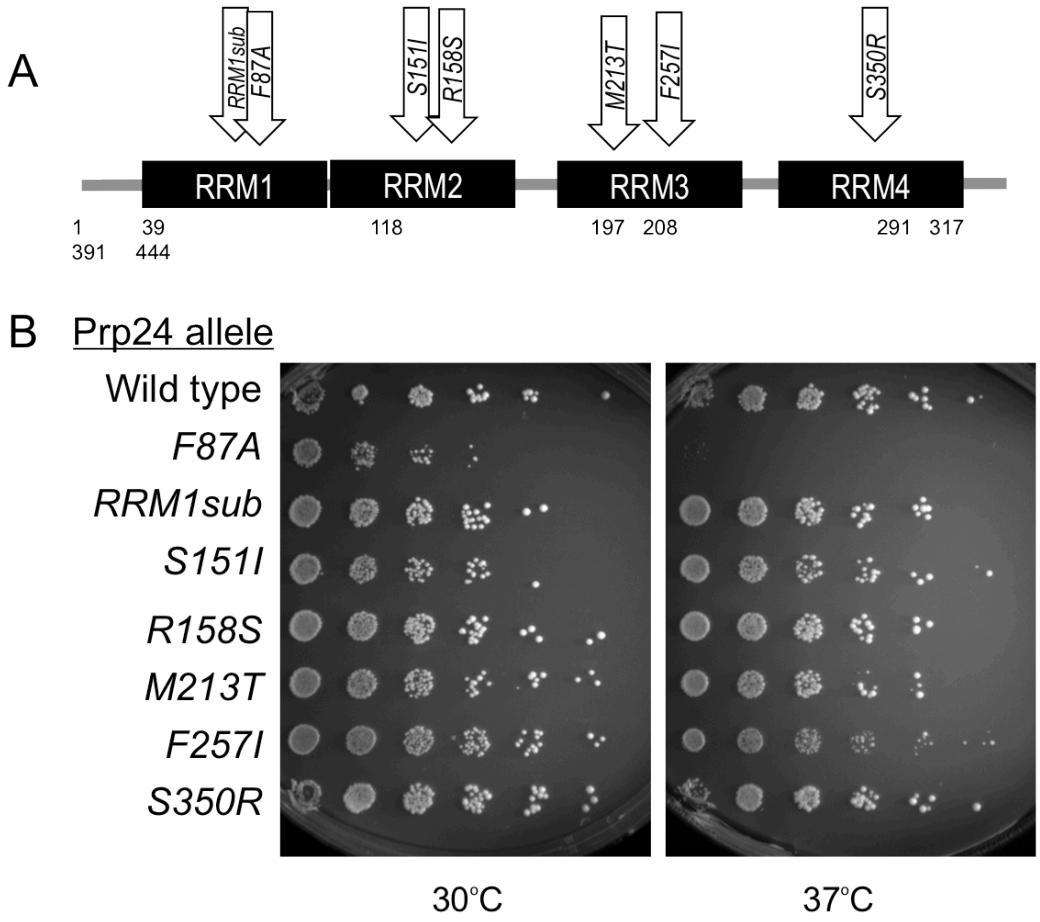

FIGURE 2: (A) RNA Recognition Motifs (RRMs) of Prp24 and mutations used in this study (B) Spotting assay showing growth of Prp24 mutant strains relative to wild-type on YPD media after 48hrs at 30 or 37 degrees Celsius.

***Prp24-F87A* has increased levels of H2Bub1**

To determine if the growth defects seen in F87A could be related to changes in H2Bub1, I performed western blots to analyze H2Bub1 levels in F87A yeast strains relative to wildtype. As seen in Figure3A, F87A mutation causes a drastic increase in total levels of H2Bub1 relative to wild-type. Moreover, this increase is specific to the F87A mutation and is not seen in any other Prp24 mutant (Figure 3B).

Given the well-studied role of Prp24 in snRNP assembly, it seemed possible that this increase may be caused by defects in splicing efficiency. However, analysis of known splicing mutants showed only a very mild increase in H2Bub1 levels, and only in the mutant with the most severe splicing defects (Figure 4A). Nevertheless, in order to confirm that the *prp24F87A* phenotypes are not due to missplicing

of a gene directly involved in regulating H2Bub1, I transformed a plasmid (Table 2) containing a cDNA copy of the SUS1 gene, the only intron-containing gene with a known role pertaining to H2B ubiquitylation. As a member of the SAGA complex, Sus1 is required for Ubp8 function and therefore could impact H2Bub1 levels if significantly misregulated (Figure 4B). However, the addition of SUS1 cDNA has no effect on the growth defects seen in *prp24F87A* (Figure 4C). Therefore, it is unlikely that splicing defects contribute significantly to the phenotype of *prp24F87A.*

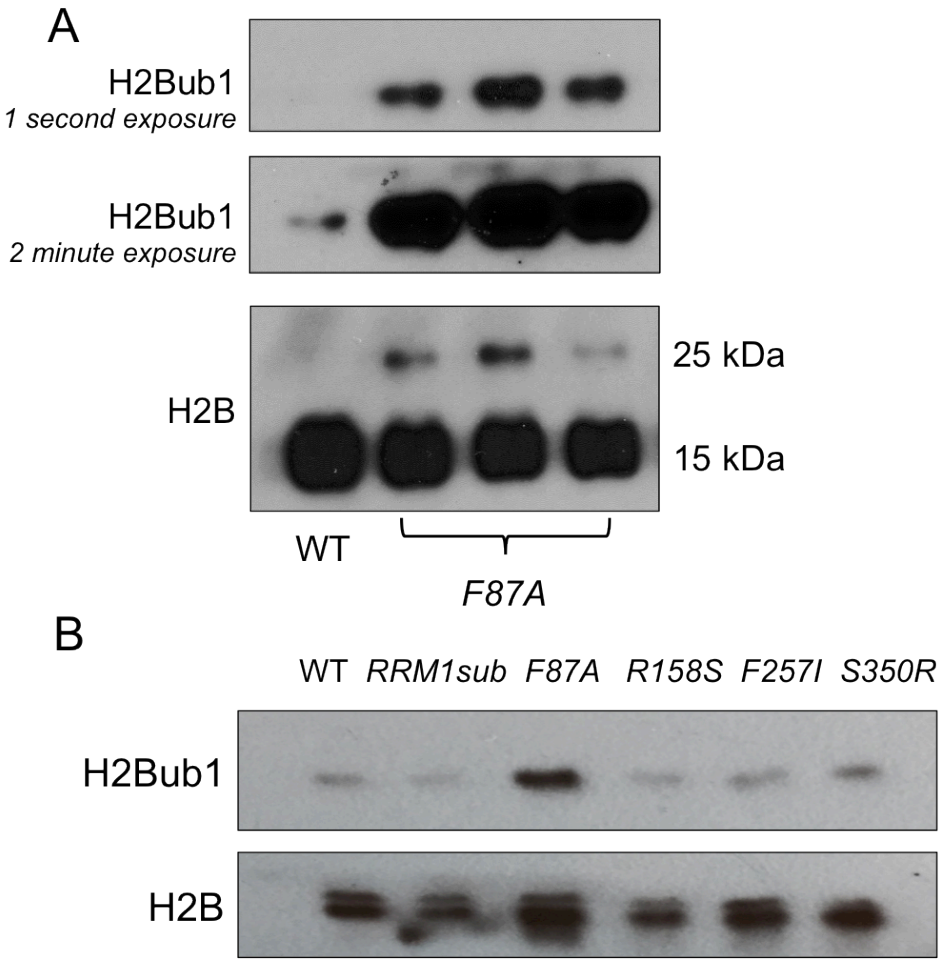

FIGURE 3: (A) Western blot comparing levels of H2Bub1. Lane 1, yeast carrying a wild-type version of Prp24. Lanes 2-4, yeast carrying a the *F87A* mutant version of Prp24. 15 kDa is the approximate running size of unmodified H2B; 25 kDa is the approximate running size of monoubiquitylated H2B. (B) Using the same antibodies as in *A*, western blot comparing levels of H2Bub1 in multiple Prp24 mutants.

Prp24-F87A affects Bre1 protein levels

Previous studies have shown H2Bub1 levels may be regulated by Bre1 stability (Wozniak & Strahl 2014). To determine if Prp24 influenced H2Bub1 through Bre1 stability, I tested the effects of F87A on Bre1 expression. When F87A cells were transformed with a FLAG-Bre1 containing plasmid, no FLAG was detected, despite significant levels seen in cells with wild-type Prp24 or other Prp24 mutations (Figure 4D). Additionally, the addition of the FLAG-Bre1 plasmid appears to decrease levels of H2Bub1 in F87A cells. Importantly, endogenous Bre1 has not been deleted from these cells. Therefore, it is possible that endogenous levels of Bre1 are much higher than levels of the tagged version. Consistent with this idea, previous studies have shown that FLAG-Bre1 levels expressed from this plasmid are much higher in a *bre1Δ* background than in wild-type yeast (BRE1). Regardless, FLAG-Bre1 levels are significantly lower in F87A, consistent with an effect of Prp24 on Bre1 regulation.

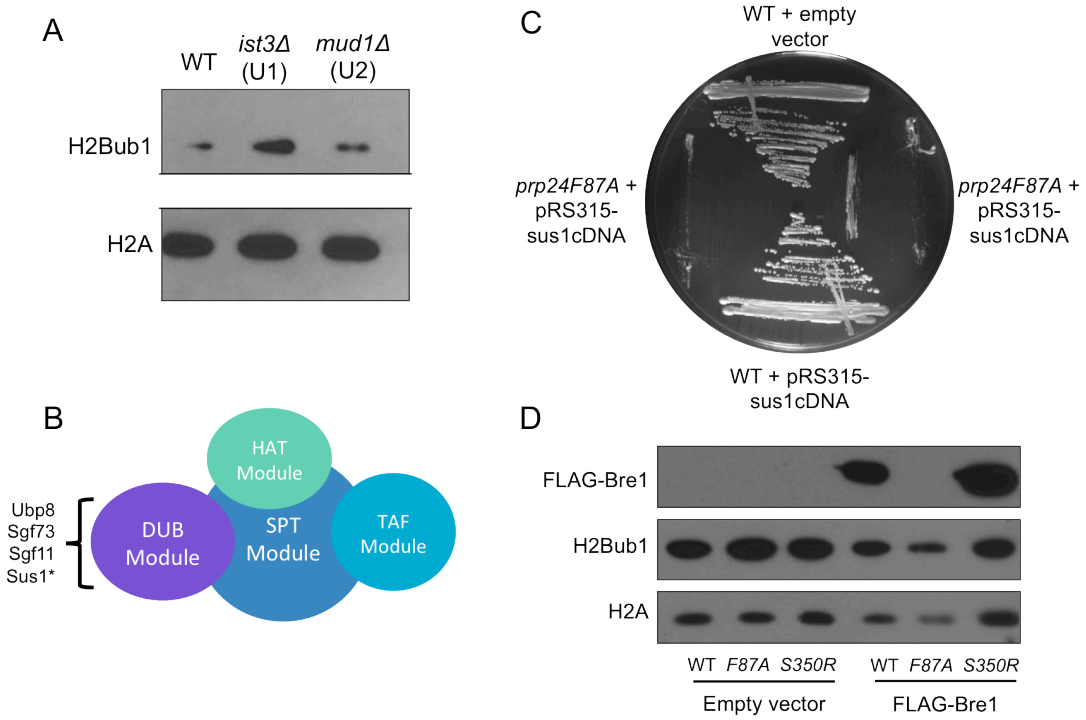

Figure 4: (A) Western blot comparing levels of H2Bub1 in yeast in which a nonessential splicing protein has been deleted. (B) Representation of the SAGA complex. Asterisk indicates the only intron-containing gene known to impact H2Bub1. (C) Yeast strains grown for 72 hours at 22°C, transformed either with pRS315 empty vector or pRS315-Sus1cDNA. (D)Western blot comparing levels of H2Bub1 and FLAG-Bre1 in strains transformed with empty vector or plasmid bearing FLAG-Bre1. Note that endogenous Bre1 has not been removed from these strains.

U6 snRNA rescues F87A growth defects

Overexpression of U6 snRNA has been reported to rescue lethality of snRNP assembly factors (Chen et al. 2006). To determine if the increase in H2Bub1 levels seen in F87A is dependent upon Prp24's interaction with U6, I overexpressed U6 snRNA in both wild-type and F87A yeast. As seen in Figure 3A, overexpression of U6 partially rescues the growth defects caused by F87A. I next sought to determine if H2Bub1 levels in F87A were also rescued by U6 overexpression. Surprisingly, overexpression of U6 reduced the levels of H2Bub1 not only in the F87A mutant, but in wild-type cells as well (Figure 3B), indicating the U6 may actually function upstream of Prp24 in regulating levels of H2Bub1.

Discussion

There remains a significant gap in understanding the links between RNA processing and histone modification. In this study, I set out to investigate the impact of a highly conserved, essential splicing factor on ubiquitylation of H2B. Using multiple mutants of Prp24, I investigated the impact of altered Prp24 function on viability and H2Bub1, uncovering a novel role for this snRNP recycling factor.

My results show that mutation of a specific residue in the first RNA Recognition Motif of Prp24, F87, significantly increases global levels of H2Bub1. This result is consistent with results obtained in HeLa cells showing that knockdown of Sart3, the human homolog, increase H2Bub1 levels. Importantly, my results indicate that this impact is restricted to a specific region of Prp24, and is not seen when other regions of the protein are mutated. Recent studies have demonstrated that Prp24 is essential for viability even when it's role in snRNP recycling is not (Burke et al. 2015). Combined with the need for RRM1 despite it's lack of interaction with U6, this finding has led to the proposal that Prp24 may have additional functions outside of U4/U6 recycling (Burke et al. 2015).

However, deletion of the N-terminal region of Prp24, including RRM1, leads to a decrease in affinity for U6 (Kwan & Brow 2005), suggesting that RRM1 function is not entirely independent of the known role of Prp24 in snRNP recycling. In support of this conclusion, I have shown that overexpression of U6 impacts H2Bub1 levels in both wild-type and *Prp24*-F87A backgrounds. It is conceivable that the F87A mutation leads to a change in levels of free U6, as has been previously seen in mutants of the

Nineteen Complex (Chen et al. 2006). If mutation of F87 were to lead to a decrease in free U6, it would be consistent with the results of Sart3 depletion in HeLa cells (Bell et al. 2002). Further experiments will be needed to test the direct effects of F87A on total levels of U6 snRNA in it's free form as well as in complex with U4 and other snRNAs.

Mutation of Prp24 at F87 also appears to have a significant impact on Bre1 levels. Previous studies have shown that Bre1 levels are highly sensitive to the ability of Bre1e to catalyze ubiquitylation of H2B, and it has been proposed that destabilization of Bre1 is an important regulator of H2Bub1 levels (Wozniak & Strahl 2014). This hypothesis offers an explanation for the absence of FLAG-Bre1 expression in *Prp24-F87A* cells. It is possible that high levels of H2Bub1 (potentially saturating amounts), lead to the degradation of Bre1. This is consistent with results showing that FLAG-Bre1 levels are lower when expressed in cells that maintain an endogenous copy of Bre1 compared to cells in which endogenous Bre1 has been deleted (Wozniak & Strahl 2014). This experiment should be repeated in a yeast strain lacking endogenous Bre1, and further experiments will be needed to determine the levels of Bre1 in other mutants with high levels of H2Bub1, such as yeast lacking Ubp8. Nevertheless, it appears evident that Prp24 does have an impact on Bre1 levels. If mutation of Prp24 impacts U6 levels, it is possible that the altered levels of Bre1 are a downstream consequence of the change in U6.

Importantly, the interaction between Prp24 and deubiquitylating enzymes has not been tested. Sart3 has been reported to associate with the human deubiquitylating enzymes Usp15 and Usp4; however, Usp4 does not appear to catalyze deubiquitylation of H2Bub1. Although Usp15 has been shown to do so *in vitro,* these results could not be replicated *in vivo* (Long et al. 2014). No deubiquitylating enzyme that is known to target H2Bub1 has been associated with Sart3 or any of its homologs.

Overall, the data presented in this study support a role for Prp24 in regulation of H2B monoubiquitylation. Although levels of U6 snRNA are likely a key factor, further studies will be needed to determine the exact role of U6 snRNA in this regulation. Importantly, these results demonstrate novel role for a well-conserved RNA-binding protein, which will be relevant for future studies investigating the function of H2Bub1 as well as its misregulation in human disease.

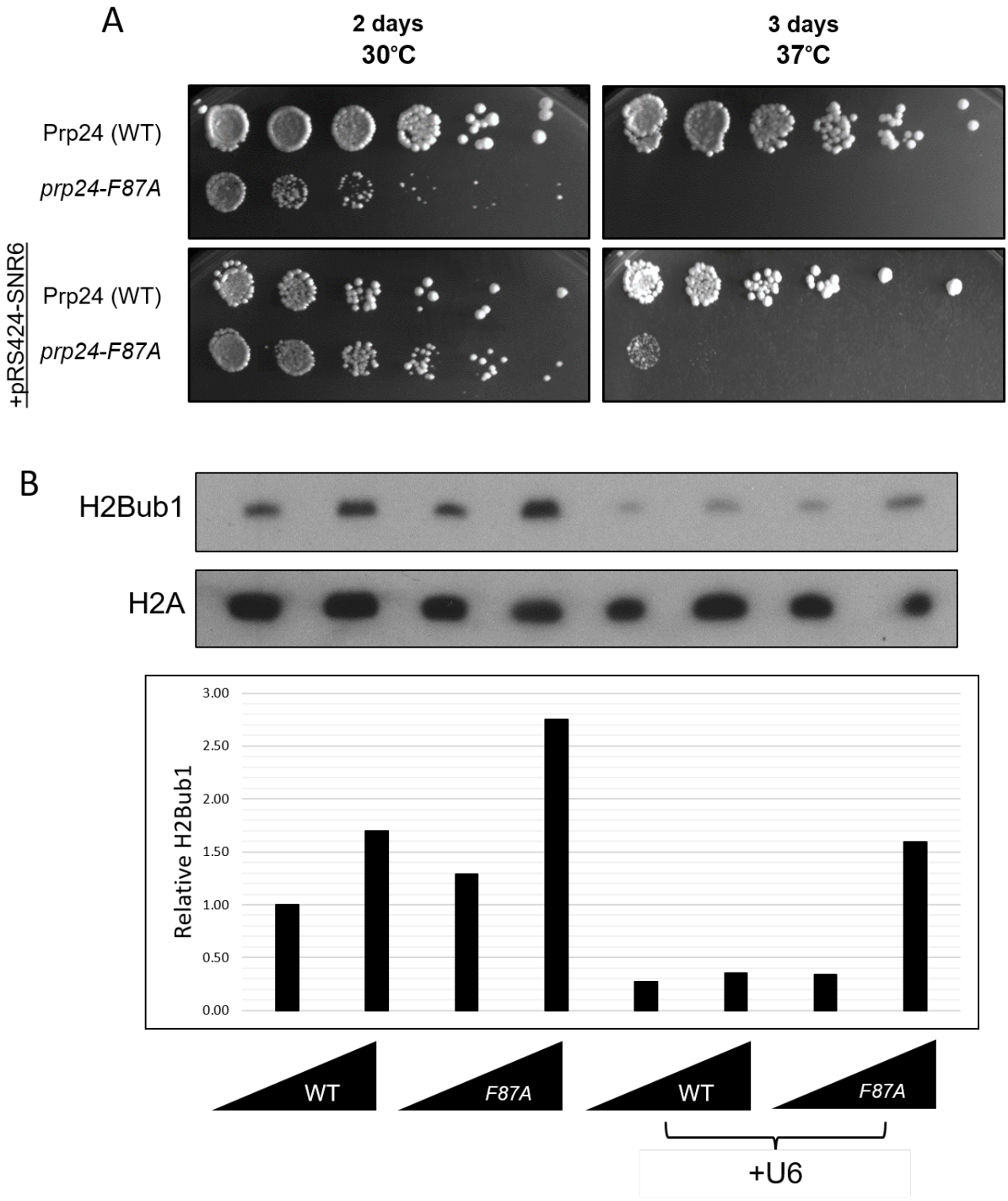

FIGURE 5: (A) Spotting assays showing growth of yeast with wildtype or mutant Prp24 with or without overexpression of U6 snRNA. (B) Top, Western blot comparing levels of H2Bub1 in the same strains shown in *A.* Bottom, image quantification. Triangles indicate increasing amount of lysate loaded onto SDS-PAGE gel before blotting.

APPENDIX: EFFECT OF ELONGATION RATE ON H2B UBIQUITYLATION

Question under investigation

Does the rate of transcription elongation affect levels of H2Bub1?

Rationale

A 2014 study (Fuchs et al. 2014) reported a correlation between local transcription rates in HeLa cells and H2Bub1 occupancy in NT2 and HTC116 cells. However, these correlation between transcriptional elongation rate and H2Bub1 levels has not been investigated in the same cell type. Additionally, it is unclear whether levels of H2Bub1 impact elongation rate or vice-versa, or if this result is mere correlation. In order to determine if altered elongation rate directly impacts levels of H2Bub1, I utilized several yeast strains carrying point mutations in the Rpb1 subunit of RNA polymerase II which have been characterized to either increase or decrease elongation rate (Braberg et al. 2013, see Table A-1 for strains used in this study).

Methods

SUMEB yeast cell extracts were prepared and western blots for H2Bub1 were performed as described previously. Spotting assays were performed as described previously.

Strains

Strains and plasmids used in this study are listed in Table A1. All strains were a gift from C. Kaplan.

Table A1: Yeast Strains

Strain	**Mutation**	**Genotype**	**Phenotype**
CKY283	wildtype	ura3-52 his3 d200 leu2 d1 or d0 trp1 d63 met15 d0 lys2-128 gal10 d56 rpb1del::natmx rpb3::tap::KlacTRP1 [pRP112 RPB1 CEN URA3]	wildtype
CKB867	G1097D	ura3-52 his3 d200 leu2 d1 or d0 trp1 d63 met15 d0 lys2-128 gal10 d56 rpb1del::natmx rpb3::tap::KlacTRP1 [pRS315 Rpb1 G1097D; LEU2 CEN ARS amp-r ColE1 Ori rpb1 10-110]	Very fast
CKB870	H1085Y	ura3-52 his3 d200 leu2 d1 or d0 trp1 d63 met15 d0 lys2-128 gal10 d56 rpb1del::natmx rpb3::tap::KlacTRP1 [pRS315 Rpb1 H1085Y; LEU2 CEN ARS amp-r ColE1 Ori rpb1 10-110]	Slow
CKB871	F1086S	ura3-52 his3 d200 leu2 d1 or d0 trp1 d63 met15 d0 lys2-128 gal10 d56 rpb1del::natmx rpb3::tap::KlacTRP1 [pRS315 Rpb1 F1086S; LEU2 CEN ARS amp-r ColE1 Ori rpb1 10-110]	Slow
CKB872	M1079R	ura3-52 his3 d200 leu2 d1 or d0 trp1 d63 met15 d0 lys2-128 gal10 d56 rpb1del::natmx rpb3::tap::KlacTRP1 [pRS315 Rpb1 M1079R; LEU2 CEN ARS amp-r ColE1 Ori rpb1 10-110]	Fast
CKB887	H1085Q	ura3-52 his3 d200 leu2 d1 or d0 trp1 d63 met15 d0 lys2-128 gal10 d56 rpb1del::natmx rpb3::tap::KlacTRP1 [pRS315 Rpb1 H1085Q; LEU2 CEN ARS amp-r ColE1 Ori rpb1 10-110]	Slow
CKB960	E1103G	ura3-52 his3 d200 leu2 d1 or d0 trp1 d63 met15 d0 lys2-128 gal10 d56 rpb1del::natmx rpb3::tap::KlacTRP1 [pRS315 Rpb1 E1103G; LEU2 CEN ARS amp-r ColE1 Ori rpb1 10-110]	Fast

Results

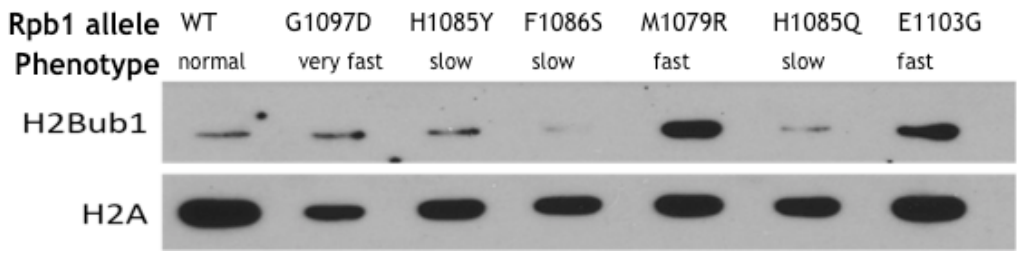

Figure A-1 Western blot showing H2Bub1 levels in various *rpb1* strains

As seen in Figure A-1, H2Bub1 levels are increased in strains which with a fast elongation phenotype and decreased or unchanged in strains with a slow elongation phenotype. The notable exception is *rpb1G1097D*, which is has the fastest elongation rate. A potential explanation for this variation is the severe sickness seen in this strain but not in the other *rpb1* mutants (Figure A-2).

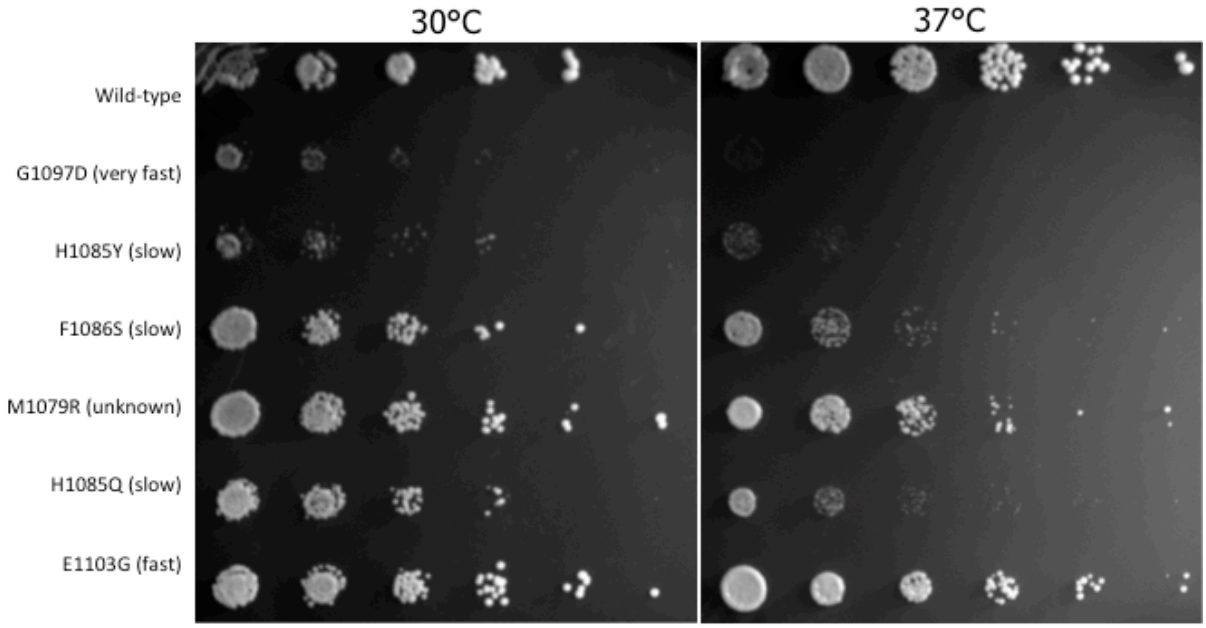

Figure A-2 Spotting assay comparing growth in *rpb1* strains after 48 hrs at indicated temperatures.

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