Role of the unconventional UBL Hub1 in pre-mRNA splicing

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Certificate of Examination

This is to certify that the dissertation titled "Role of the unconventional UBL Hub1 in pre-mRNA splicing" submitted by Ms. B Nivedha (Reg. No. MP18002) for the partial fulfilment of MS degree program of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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Dated: May 20, 2021

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Shravan Kumar Mishra at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

(Candidate)

Dated: May 20, 2021

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Shravan Kumar Mishra

(Supervisor)

Acknowledgement

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Contents

Abstract

Pre-mRNA splicing by the spliceosome is one of the steps where regulation of gene expression occurs. Various ubiquitin-like proteins (UBLs) have been shown to regulate pre-mRNA splicing. The UBL Hub1 is known to play a role in alternative splicing in *Saccharomyces cerevisiae* through its well-known surfaces. This study shows that the recently identified novel surface of Hub1 in *Schizosaccharomyces pombe* plays a significant role in cell growth and splicing. The various approaches of bioinformatic analysis, genetics and splicing assays suggest a possible link between Hub1 and transcription. This study also shows that Hub1 selectively modifies the spliceosome and that it might play a role in the transition of the spliceosome. Therefore, this study addresses the mechanism and function of Hub1 in *Schizosaccharomyces pombe*.

Chapter 1

Introduction

1.1 Transcription

Transcription is the first step in gene expression where RNA is produced using DNA as the template and is carried out by a large multi-subunit complex called RNA polymerase. In eukaryotes, the protein-coding genes are transcribed into messenger RNA (mRNA) by RNA polymerase II whereas RNA polymerase I transcribes rRNA genes and RNA polymerase III catalyzes the transcription of 5S rRNA and tRNA.

Initiation of transcription begins with the recognition of transcription start site (TSS) in the promoter, recruitment of RNA polymerase II and general transcription factors to TSS followed by promoter clearance. TFIID is the first general transcription initiation factor that recognizes and binds to the core promoter element through its TATA binding protein (TBP) component (Patel et al., 2020). TFIIA then assembles with TFIID and stabilizes the TBP-DNA interactions (ROEDER, 1996). This is followed by sequential recruitment of other general transcription initiation factors TFIIB, TFIIF, the RNA polymerase II and TFIIE, TFIIH along with additional initiation factors resulting in the formation of a complete pre-initiation complex (PIC) (Weinmann, 1992). After the assembly of complete pre-initiation complex, ATP dependent activation of PIC results in the formation of an open complex and the factors TFIIE and TFIIH cause promoter melting facilitating transcription initiation and transition into elongation phase (ROEDER, 1996; Conaway & Conaway, 1993).

Transcription elongation is a highly regulated process involving several elongation factors. Some of the elongation factors like ELL, Elongin, TFIIF promote transcriptional elongation by suppressing transient pauses (J. W. Conaway et al., 2000). In addition to elongation factors, SWI/SNF family members facilitate transcription elongation by modifying chromatin structure (Reines et al., 1999). The regulatory elements in promoter influence the rate of transcription by providing a platform for efficient recruitment of elongation factors (Montanuy et al., 2008).

The carboxy-terminal domain (CTD) of Rpb1, the largest subunit of RNA pol II acts as a scaffold for many factors and contains heptad repeats whose phosphorylation plays a major role in regulating transcription elongation (Phatnani & Greenleaf, 2006). The pattern of phosphorylation of CTD changes as RNA pol II progresses through transcription cycle which

facilitates the recruitment of different factors at different stages of transcription elongation (Hsin et al., 2014; Harlen & Churchman, 2017). After the completion of transcription, the RNA pol II disassembles and transcription is terminated.

1.2 Transcription is coupled to RNA processing events

The precursor mRNA (pre-mRNA) synthesised by RNA pol II is not yet ready for translation and has to undergo further processing to become a mature mRNA. It undergoes 5' capping, splicing and 3' polyadenylation in the nucleus before it is transported to the cytoplasm. It is well known that processing of pre-mRNA occurs co-transcriptionally and its functional coupling to RNA pol II is required for efficient and co-ordinated gene expression (Figure 1A) (Lee & Tarn, 2013). The CTD of Pol II plays a key role in coupling transcription and RNA processing. The capping enzyme in *Saccharomyces cerevisiae* (*S. cerevisiae*) binds to phosphorylated CTD of early transcribing pol II and carries out pre-mRNA 5' capping (Martinez-Rucobo et al., 2015). Similar to 5' capping, the phosphorylated CTD of RNA pol II provides a platform for the recruitment of several splicing factors and 3' end processing machinery, thereby coupling pre-mRNA processing with transcription.

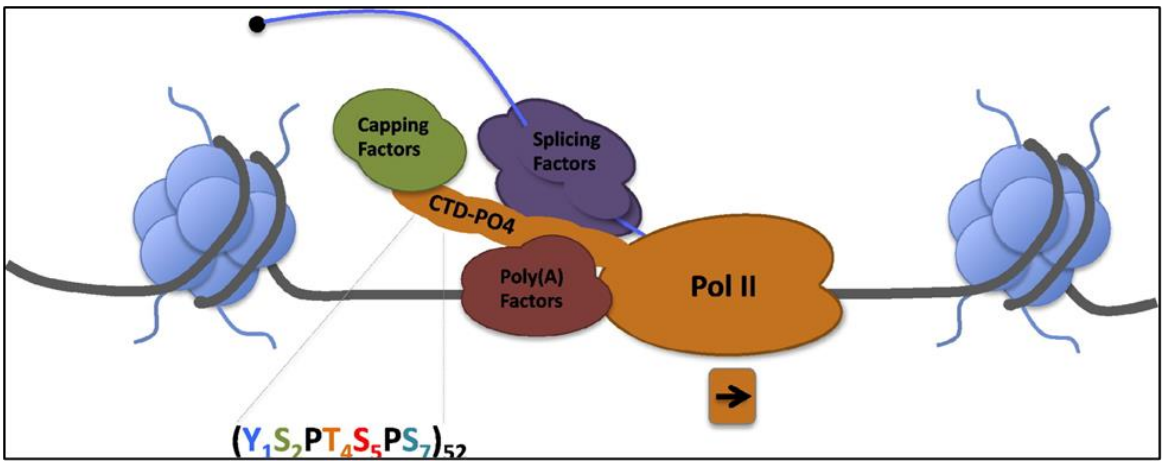

Figure 1A: Schematic showing co-transcriptionality of pre-mRNA processing (Figure source: Saldi et al., 2016)

1.3 Pre-mRNA splicing and the spliceosome

The protein-coding regions of eukaryotic genes are interrupted by non-coding regions called introns. The process of removal of non-coding introns and joining of exons is called pre-mRNA splicing and is carried out by the spliceosome. Many intronic sequence elements like 5' splice site, branch point, 3' splice site and polypyrimidine tract are required for pre-mRNA splicing. Splicing takes place via two sequential transesterification reactions (Shi, 2017). In the first reaction, the 2' OH of the branchpoint adenosine attacks the 5' splice site resulting in a covalent linkage of the 5' phosphate of the 5' splice site with the 2' oxygen of the branch point and a 5' exon with free 3' OH group. In the second reaction, the 3' OH of the free 5' exon attacks the 3' splice site resulting in exon-exon ligation and the release of an intron-lariat.

The spliceosome is a highly dynamic ribonucleoprotein complex which carries out the process of pre-mRNA splicing. It is composed of five U-rich small nuclear RNA (U snRNA) and U-snRNA specific proteins called U small nuclear ribonucleoproteins (U snRNPs). During sequential assembly of the spliceosome, it undergoes extensive structural rearrangements during each step. The U1 snRNP is recruited to the 5' splice site resulting in the formation of E-complex followed by the assembly of U2 snRNP at the branch point forming A complex (Plaschka et al., 2018). Subsequently, the tri-snRNP U4/U6.U5 joins the spliceosome forming a pre-catalytic B complex. Then U1 and U4 snRNPs dissociate to form the B complex. Further rearrangements result in B^{act} complex which gets converted to B* complex by Prp22 and further to form the catalytic C complex which carries out the first transesterification reaction (Wan et al., 2019). Formation of C* complex by Prp16 carries out exon ligation by second transesterification reaction resulting in post-splicing P complex which then gets disassembled for further rounds of splicing (Fica & Nagai, 2017).

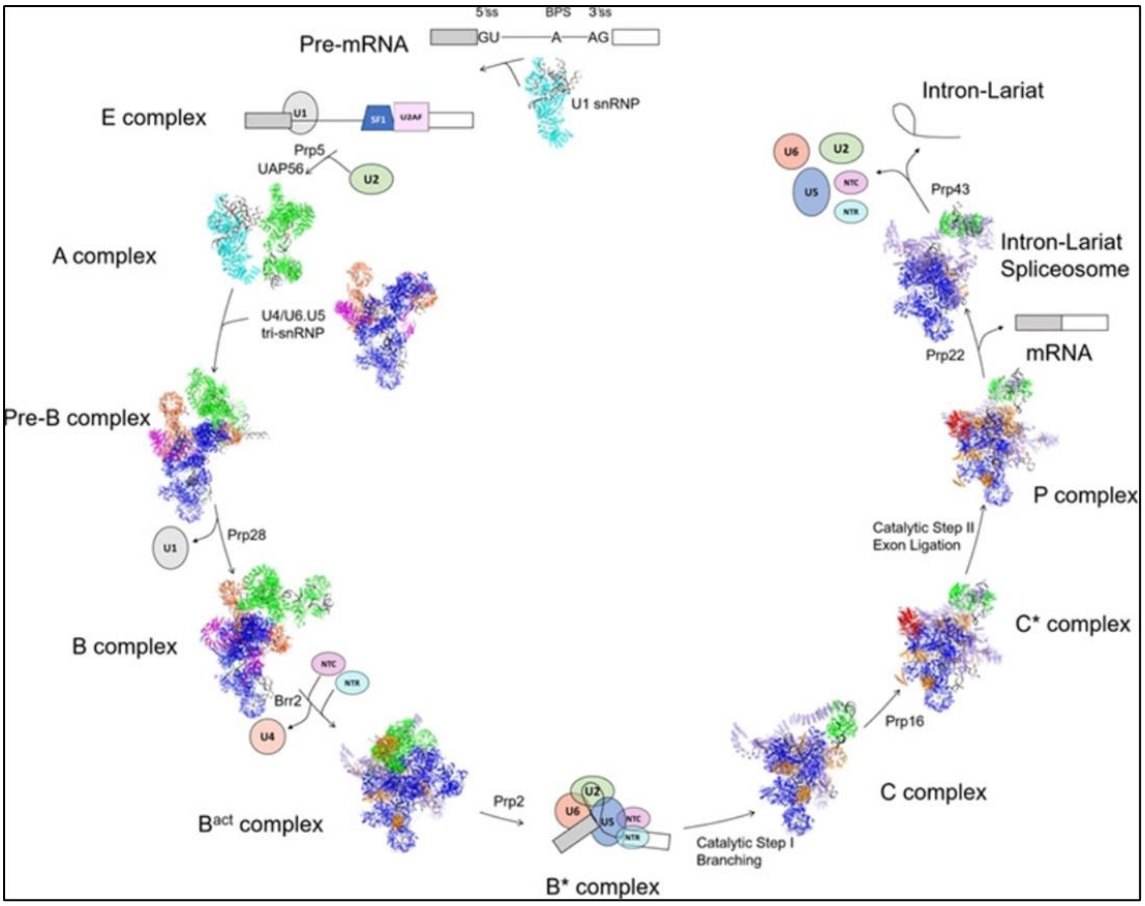

Figure 1B: Sequential stages of assembly of the spliceosome (Figure source: Zhang et al., 2019)

1.4 Co-transcriptional splicing

The process of splicing of pre-mRNAs when the transcription is ongoing is called co-transcriptional splicing whereas splicing of pre-mRNAs post transcription is called post-transcriptional splicing. Most of the splicing events in eukaryotes take place co-transcriptionally (Brugiolo† et al., 2013). Various studies have reported several links between splicing and transcription during co-transcriptional splicing. The Prp19 complex, a core component of the spliceosome also plays a role as a transcription elongation factor and is required for the occupancy of TREX complex at transcribing genes (Chanarat et al., 2011). SR proteins are a family of RNA binding proteins involved in splicing. SRSF2, a unique SR protein has been shown to function as a transcription activator (Ji et al., 2013). The phosphorylated CTD of RNA pol II acts a platform for recruiting several splicing factors (Alexander & Beggs, 2010). Also, it has been shown that U1 snRNP at 5' splice site can recruit general transcription

factors suggesting the existence of bi-directional coupling between transcription and splicing (Damgaard et al., 2008). It has been shown that rapid and efficient co-transcriptional splicing enhances gene expression in mammals (Reimer et al., 2021). Also, alternative splicing is affected in mammalian cell lines by a slow RNA pol II (de la Mata et al., 2003). All these observations and further evidences suggest that coupling between transcription and splicing acts as a gene regulatory mechanism.

1.5 Ubiquitin and Ubiquitin-like modifiers

Post-translational modification of proteins by covalent attachment of ubiquitin usually targets the proteins to degradation by proteasome. Ubiquitination has also been shown to play a regulatory role in signalling mechanisms that control diverse physiological processes (Sun & Chen, 2004). Ubiquitin-like proteins (UBLs) are a class of proteins that share three-dimensional structure (β-grasp fold) with ubiquitin and act as modifiers similar to that of ubiquitin (Jentsch & Pyrowolakis, 2000). Some of the UBLs include small-ubiquitin like modifier (SUMO), NEDD8, ATG12 and URM1. UBLs are synthesized as inactive precursors which are processed by UBL-specific proteases and get covalently conjugated to proteins via their C-termini (Jentsch & Pyrowolakis, 2000). The UBL conjugation pathway is similar to ubiquitination which involves the sequential action of the ubiquitin-activating enzymes (E1), ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3) resulting in the attachment of ubiquitin to proteins.

UBLs have been reported to act as regulators of various biological processes in eukaryotes. SUMO modification influences protein-protein interactions, protein stability, subcellular localization, substrate recognition thereby regulating several cellular (Müller et al., 2001; Wilkinson & Henley, 2010). ISG15 is another UBL whose function has been implicated in antiviral immune response (Perng & Lenschow, 2018). Atg8 and Atg12, UBLs which are members of ATG family play an important role in the initial stages of autophagosome formation (Geng & Klionsky, 2008). Function of UBLs has also been reported in pre-mRNA splicing. Sde2, a protein with ubiquitin fold gets processed by UBL-specific proteases after which the C-terminal domain Sde2-C gets incorporated into the spliceosome and acts as an intron-specific splicing factor (Thakran et al., 2017). Hub1 is another UBL which is involved in alternative splicing of *SRC1* in *S. cerevisiae* by promoting the usage of non-canonical 5' splice site (Mishra et al., 2011).

1.6 The unconventional UBL Hub1

Among the UBLs, Hub1 is an unconventional UBL lacking the C-terminal di-glycine motif and binds to proteins non-covalently (Luders et al., 2003). In *S. cerevisiae*, Hub1 is non-essential but it is required for the alternative splicing of SRC1 and non-canonical splice site usage of pre-mRNAs (Mishra et al., 2011). Hub1 binds to Snu66, a protein of U4/U6. U5 tri-small nuclear ribonuclear protein (tri-snRNP) through its D22 surface to promote alternative splicing (Wilkinson et al., 2004). Also, Hub1 binds to the DEAD-box helicase Prp5 via H63 surface, stimulates its ATPase activity and promotes efficient splicing (Karaduman et al., 2017). Further, it has also been shown that activation of spliceosome by Hub1 results in increased splicing efficiency at the cost of reduced splicing fidelity. In *Schizosaccharomyces pombe* (*S. pombe*) and higher eukaryotes where splicing is predominant, Hub1 is essential for viability (Luders et al., 2003, Mishra et al., 2011, Ammon et al., 2014). A temperature-sensitive structural mutant of Hub1, namely *hub1-1* (*hub1 I42S*) was identified in *S. pombe* and splicing defects have been observed in this mutant. This suggests the possible role of Hub1 in pre-mRNA splicing in *S. pombe*.

Objective

As the role of Hub1 in pre-mRNA splicing in higher eukaryotes remains unexplored, this study focusses on identifying the function and mechanism of Hub1 in pre-mRNA splicing in the intron rich eukaryote *S. pombe*.

Chapter 2

Experimental methods

2.1 Strains and plasmids

S. pombe strains and plasmids used in this study are enlisted in Appendix Table S1 and S2 respectively. The *S. pombe* strains were stored at -80° C by mixing saturated cultures grown in YEL at 30°C with sterile 50% glycerol (v/v) in the ratio of 1:3 and later revived by streaking them on YES plates. Preparation of *S. pombe* competent cells and transformation was done following the published protocols (Knop et al., 1999). Briefly, 20 µL of competent cells and 2 µL of plasmid were mixed with filter-sterilized 40% PEG (six times the total volume) and incubated at 30°C for at least 30 mins. Following the incubation, heat shock at 42°C was given for 5 mins (for temperature-sensitive strains, 1 min heat shock was given), plated on selection media and incubated until growth appeared.

2.2 Growth assay

Freshly streaked cells were dissolved in sterile water and OD_{600nm} was measured. Cells corresponding to 1 OD_{600nm} were taken and subjected to five-fold serial dilution in a microtiter plate. Then spotting was done on selected agar plates and incubated at different temperatures until growth was observed.

2.3 RNA isolation and RT-PCR

RNA isolation and cDNA synthesis were done following the published protocol (Inada & Pleiss, 2010). Logarithmically growing cells corresponding to 5 OD_{600nm} were harvested by filtration after 1 hour of heat shock at 37°C. Total RNA was isolated by hot phenol method. The harvested cells were mixed with acid phenol: chloroform, AES buffer and vortexed vigorously once every minute for 7-8 minutes in a water bath set at 65°C. Following the cell lysis, the cell suspension was incubated on ice for 5 mins and the entire suspension was transferred to a pre-spun 15 mL phase-lock gel tubes. The tubes were centrifuged at 3000g for 5 mins at 4°C followed by addition of phenol: chloroform: isoamyl alcohol and centrifugation.

Then, chloroform was added and centrifuged. The aqueous phase was transferred to new 15 mL falcons followed by the addition of sodium acetate and isopropanol and left at -20°C for at least 20 mins. Then, 2 mL of the slurry was centrifuged at maximum speed for 20 mins at 4°C and the RNA pellet was washed twice with 70% ethanol. The RNA pellet was dried in a vacuum concentrator and resuspended in nuclease free water. 10 µg of RNA was subjected to DNase I treatment for 15-20 mins at room temperature and then Zymospin column was used to obtain RNA. 2 µg of DNase-treated RNA was used for cDNA synthesis using random hexamers and Reverse Transcriptase. The synthesised cDNA was used for further target specific RT-PCRs.

2.4 Immunoprecipitation (large scale)

The assay for immunoprecipitation of in vivo spliceosomal complex was done as described previously (Mishra et al., 2011). Logarithmically growing cells in YEL were shifted to 37°C for 3 hours when the OD_{600nm} was approximately 0.7-0.8. Then, cells corresponding to 1600 OD_{600nm} were harvested by centrifugation at 3000 rpm for 10 mins at 4°C. The supernatant was discarded and the cell pellet was resuspended in cell lysis buffer with PMSF, protease inhibitor cocktail and flash-frozen in liquid nitrogen and stored at -80°C.

Cell lysis was done by mechanical grinding with liquid nitrogen followed by thawing. Then, the total cell lysate was pre-cleared twice by centrifuging at 10,000g for 10 mins at 4°C. 2% of the supernatant was taken as input and heated at 65°C for 10 mins with HU buffer. After pre-clearing, the supernatant was transferred to a new 15 mL falcon and immunoprecipitation was done using HA beads (200 µL) for 6 hrs on a slow rotor (10 rpm) at 4°C. Following immunoprecipitation, the beads were subjected to following washes at 3000 rpm for 2 mins at 4°C – 1X with diluted lysis buffer with PMSF and protease inhibitor cocktail, 3X with lysis buffer with only 1% triton X-100 and a final 1X wash with lysis buffer without triton X-100. Residual supernatant was completely removed by vaccusip and the immunoprecipitated proteins were eluted in 30 µL HU buffer and heated at 65°C for 10 mins. 15 µL of the protein sample was subjected to mass spectrometry.

2.5 Chromosomal deletion

To make chromosomal deletion of a gene, the published protocol in Janke et al., 2004 was followed. The Kan MX6 cassette which confers resistance against G418 antibiotic was flanked with the promoter and terminator sequences of the gene to be deleted. Then, the cassette with the promoter and terminator sequences was digested overnight with NotI and transformed in freshly made *S. pombe* competent cells. The transformation was followed by revival for 6-8 hrs in YEL at 30°C shaker and plating on YES+G418 plates.

Chapter 3

Results

3.1 The novel surface of Hub1 is required for cell growth and pre-mRNA splicing

It is known that Hub1 binds to the tri-snRNP protein Snu66 through its D22 surface and the DEAD-box helicase Prp5 via its H63 surface in *S. cerevisiae* and both these surfaces are conserved in higher eukaryotes. Dr. Kiran Kumar in collaboration with Dr. Ranabir Das had identified a novel surface of *S. pombe* Hub1 containing R9 and R41 residues (referred to as Hub1 R9R41) by using the approaches of NMR, mutagenesis and complementation in *S. pombe* Hub1 knockout strain. *hub1-R9A R41A* mutant is a temperature-sensitive mutant similar to *hub1-1* as it grows at the permissive temperature of 30°C but not at the non-permissive temperature of 37°C. Unlike *hub1-1*, *hub1-R9A R41A* mutant is not a structural mutant. Structure of *S. pombe* Hub1 was predicted using the structure prediction program i-TASSER and the three surfaces of Hub1 have been marked among which the novel surface (R9R41) is located at the C-terminus (Figure 3.1A) (Yang et al., 2014).

In order to address the significance of this novel surface of Hub1 in *S. pombe*, a fivefold serial dilution spot assay was done using all the Hub1 mutants (*hub1-1*, *hub1-D22A*, *hub1-H63L*, *hub1-D22A H63L*, *hub1-R9A R41A*) to check for any growth phenotype. *hub1-D22A* and *hub1-R9A R41A* mutants showed growth sickness, *hub1-D22A H63L* mutant was slightly sick at 30°C whereas the other Hub1 mutants did not show any phenotype (Figure 3.1B). As previously reported by Dr. Kiran Kumar, *hub1-R9A R41A* mutant indeed showed temperature sensitivity like *hub1-1* whereas other Hub1 mutants were not temperature-sensitive (Figure 3.1B). The growth phenotype shown by this mutant suggests that the novel R9R41 surface of Hub1 is important for its growth.

To further understand the role of Hub1 in pre-mRNA splicing, a genome-wide splicing-sensitive microarray was done in *hub1-1* by Dr. Shravan Kumar Mishra in collaboration with Dr. Jeffrey Pleiss. It was observed that the splicing of many genes was affected in this mutant as there was an accumulation of intron-containing transcripts compared to wild type (WT) (Figure 3.1C). Further validation of the microarray data was done by RT-PCR assay for all the Hub1 mutants. All the Hub1 mutant strains along with WT as a control were grown at 30°C

and shifted to 37°C for 1 hour. Following the harvest, total RNA was isolated, cDNA was synthesized and RT-PCR was done using target specific primers to capture both intron-containing transcripts and mature transcripts. *hub1-D22A*, *hub1-H63L*, *hub1-D22A H63L* mutants showed mild splicing defects whereas *hub1-1* and *hub1-R9A R41A* mutants showed strong splicing defects for many targets where only the intron-containing transcripts and no mature transcripts was seen (Figure 3.1D). The strong splicing defects seen in *hub1-R9A R41A* mutant suggest the essential role of this novel surface in pre-mRNA splicing.

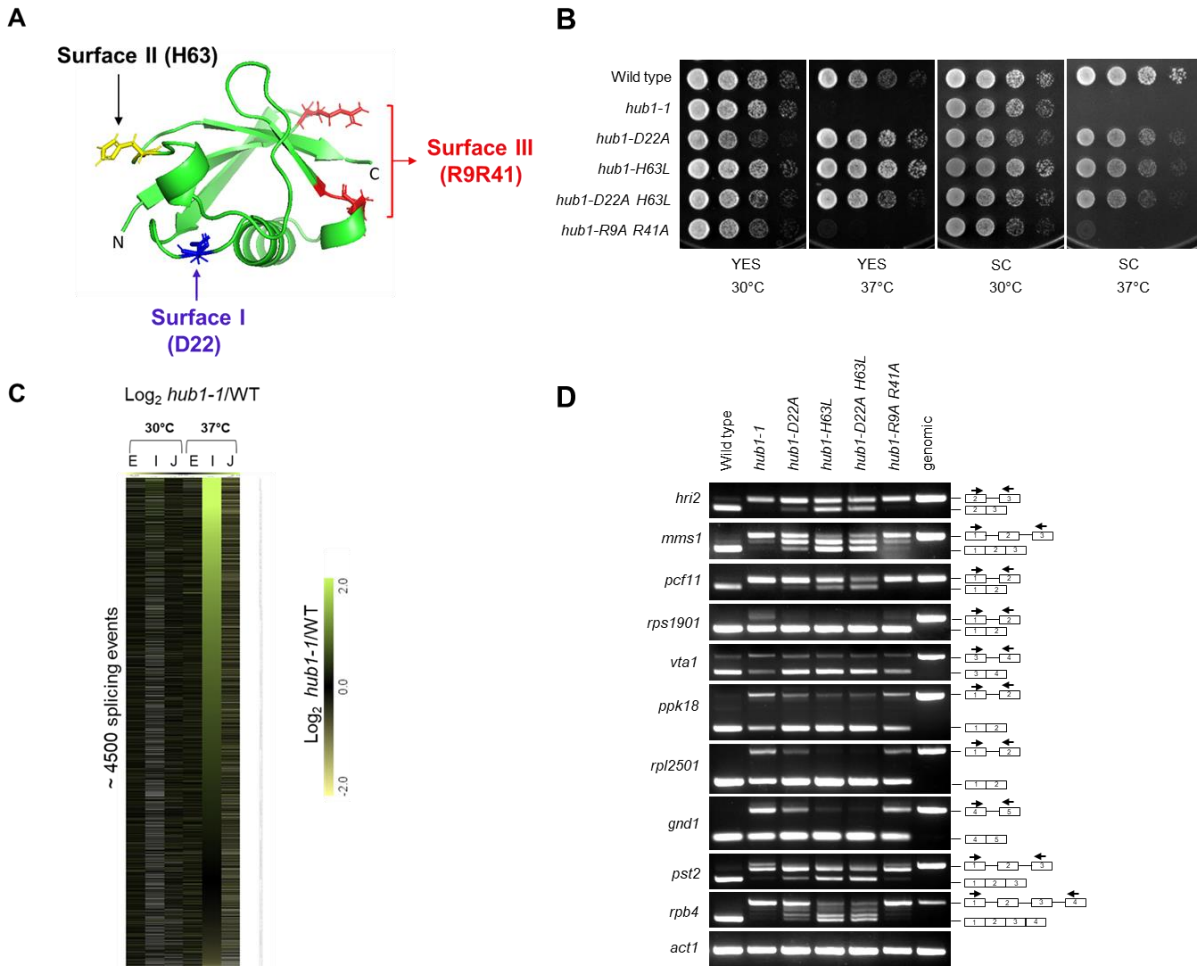

Figure 3.1: The R9R41 surface of Hub1 plays an important role in cell growth and pre-mRNA splicing

A) The predicted structure of *S. pombe* Hub1 with the following three surfaces of Hub1 highlighted – surface I comprising of D22 residue, surface II formed by H63 residue and R9R41 residues forming surface III at the C-terminus.

B) Growth phenotype of *S. pombe* WT, *hub1-1* (structural mutant), *hub1-D22A* (surface I mutant), *hub1-H63L* (surface II mutant), *hub1-D22A H63L* (surface I + surface II mutant) and *hub1-R9A R41A* (surface III mutant). Fivefold serial dilution spot assay

was done on YES (rich media) and SC (defined media) agar plates and incubated at 30°C and 37°C.

C) Microarray heatmap shows $\log_2$ *hub1-1*/WT ratio of total transcripts (E), intron-retained transcripts (I) and mature transcripts (J). Increasing value of $\log_2$ *hub1-1*/WT ratio indicates accumulation of intron-retained transcripts. (Data from Dr. Shravan Kumar Mishra).

D) Semi-quantitative RT-PCR shows an accumulation of intron-retained transcripts in Hub1 mutants for many target genes. 37°C temperature shift for 1 hour was done before the harvest. cDNA synthesised from total RNA isolated from WT and Hub1 mutants was analysed by PCR using target specific primers. The blocks with the numerical indicate exon numbers and the arrows depict the primers used in this assay.

3.2 Hub1 targets have a common characteristic feature

To address whether Hub1 dependent targets have any common characteristic features, a bioinformatic analysis was done by Dr. Kiran Kumar in collaboration with Dr. Arashdeep Singh and all the possible features of an intron and a transcript were analyzed. Interestingly, they observed a negative correlation between the synthesis time of those transcripts dependent on Hub1 for splicing and the splicing defects in *hub1-1* (Figure 3.2A). This suggests the possibility that those transcripts that are synthesized faster are dependent on Hub1 for splicing.

For validation of the bioinformatic analysis, *mug37* which has a synthesis time of 90 minutes was chosen. The following two constructs – *mug37* with its own promoter and terminator and *mug37* in an over-expression vector with a thiamine-repressible promoter (nmt 81X) were transformed in WT, *hub1-1* and *hub1-R9A R41A* strains. Thiamine-repressible promoter was chosen as it is known to increase the transcription efficiency (Belén Moreno et al., 2000). The splicing defects were then monitored for the transformants at 30°C and 37°C (1 hour). The transformants containing the construct of *mug37* with its own promoter showed an accumulation of intron-retained transcripts of *mug37* in all the strains including WT suggesting that its splicing is independent of Hub1 (Figure 3.2B). Whereas the transformants containing the construct of *mug37* under thiamine-repressible promoter showed an accumulation of intron-retained transcripts only in *hub1-1* and *hub1-R9A R41A* indicating that splicing of *mug37* transcripts now becomes dependent on Hub1 (Figure 3.2B). Thus, when a slowly synthesized gene like *mug37* is placed under a strong promoter which results in efficient transcription, the splicing of that transcript becomes dependent on Hub1 validating the bioinformatic analysis.

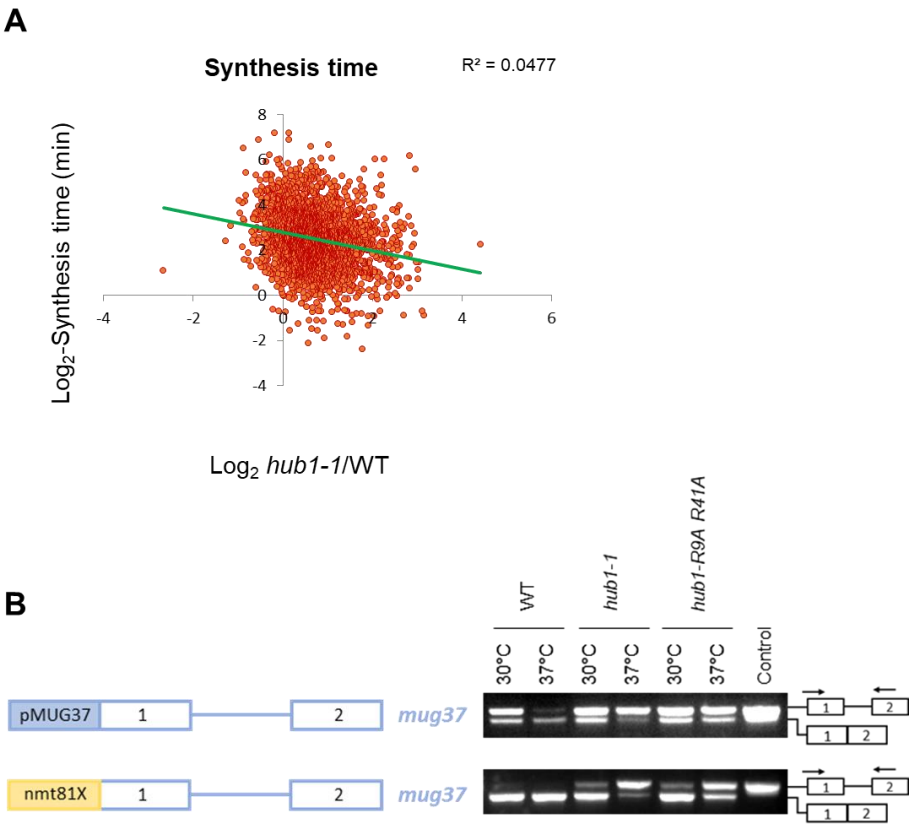

Figure 3.2: Validation of the correlation between synthesis time of transcripts and splicing defects in Hub1 mutants

A) Correlation graph shows a negative correlation between synthesis time of transcripts and splicing defects in *hub1-1*. (Data from Dr. Kiran Kumar).

B) Semi-quantitative RT-PCR shows an accumulation of intron-retained transcripts in both WT and Hub1 mutants when transformed with *mug37* with its own promoter and an accumulation of intron-retained transcripts only in Hub1 mutants when transformed with *mug37* with nmt81X promoter. 37°C temperature shift for 1 hour was done before harvest. The blocks with the numerical indicate exon numbers and the arrows depict the primers used in this assay.

3.3 Hub1 shows genetic interaction with the transcription elongation factor Spt4

To further confirm the observation that Hub1 is required for splicing of those genes that are synthesized faster, a genetic approach was taken. The transcription elongation factors Spt4/5 form a protein complex, bind to RNA pol II and play a major role in regulating transcription elongation (Klein et al., 2010; Hartzog & Fu, 2013). Spt4 and Spt5 mutants have been shown to be elongation-defective (Hartzog & Fu, 2013; Rondon, 2003).

We wanted to see if there will be a rescue in the phenotype of Hub1 mutants upon slowing down transcription elongation. Booth et al., 2016 has reported a global reduction in transcription elongation rate in *Δspt4* in *S. pombe* suggesting that the genes are synthesised slowly. Hence, the double mutants (*hub1-1 Δspt4* and *hub1-R9A R41A Δspt4*) were generated and checked for their phenotype. Surprisingly, the double mutants showed a synthetic sickness at 30°C suggesting a negative genetic interaction between Hub1 and the transcription elongation factor Spt4. However, there was no rescue of temperature-sensitivity of Hub1 mutants at 37°C. Thus, the genetic interaction between Hub1 and Spt4 suggests the possibility of a link between Hub1 and transcription.

Further, we also wanted to see if there is a rescue of splicing defects in the double mutants using splicing assay. Interestingly, there was a mild rescue of splicing defects in the double mutant *hub1-R9A R41A Δspt4* and a very slight rescue in the double mutant *hub1-1 Δspt4*. This observation possibly suggests that splicing becomes less dependent on Hub1 when the elongation rate is reduced.

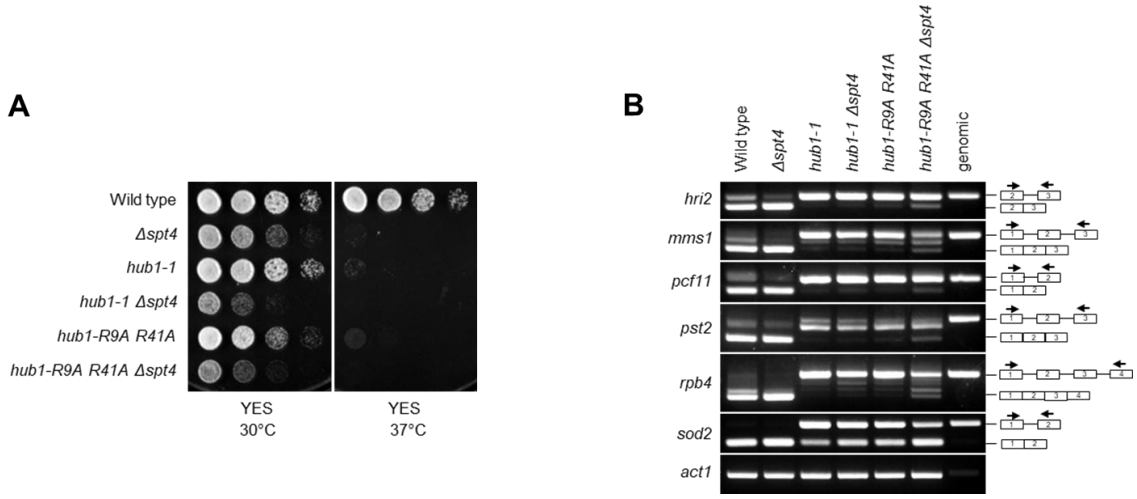

Figure 3.3: Genetic interaction between Hub1 and Spt4

A) The double mutants - *hub1-1 Δspt4* and *hub1-R9A R41A Δspt4* show synthetic sickness at 30°C on YES and the rescue of temperature sensitivity is not observed in the double mutants at 37°C on YES.
B) Semi-quantitative RT-PCR shows a mild rescue of splicing defects in the double mutant *hub1-R9A R41A Δspt4* for all the chosen targets and a very slight rescue of splicing defects in the double mutant *hub1-1 Δspt4* for some of the targets. Rescue of splicing defects refers to the increased presence of cDNA band. 37°C temperature shift for 1 hour was done before harvest. The blocks with the numerical indicate exon numbers and the arrows depict the primers used in this assay.

3.4 Rpb10 overexpression rescues the temperature-sensitivity and splicing defects of Hub1 mutants

Yashiroda & Tanaka, 2004 had identified Snu66 and Rpb10 as high copy suppressors of the *hub1-1* temperature sensitive mutant. Snu66, a protein of tri-snRNP complex is an interactor of Hub1 in *S. pombe* and the rescue of *hub1-1* by over-expression of Snu66 could be possibly due to stabilization of the structure of the mutant Hub1 (Wilkinson et al., 2004). Rpb10 is a common subunit of the RNA polymerases I, II and III. This subunit is essential for viability in *S. pombe* and it has been reported that it is involved in the assembly of RNA polymerase (Woychik & Young, 1990; Wild & Cramer, 2012). The fact that a subunit of RNA polymerase rescues the phenotype of *hub1-1* is interesting. Unlike Snu66, Rpb10 does not interact with Hub1 in yeast two-hybrid (Y2H) assay (Dr. Shravan Kumar Mishra, unpublished) and thus it is unlikely that Rpb10 rescues *hub1-1* by stabilizing the structure of mutant Hub1.

In order to check if over-expression of Rpb10 rescues *hub1-R9A R41A* like *hub1-1*, it was expressed under nmt81X promoter in WT, *hub1-1* and *hub1-R9A R41A*. It was indeed observed that the rescue of *hub1-R9A R41A* was better than that of *hub1-1*. This suggests that overexpression of Rpb10 rescues *hub1-R9A R41A* too.

Further, we also wanted to check if there is a rescue of splicing defects upon Rpb10 overexpression in the mutants. A splicing assay was done using WT and Hub1 mutant strains expressing empty vector and Rpb10. A mild rescue of splicing defects was seen in *hub1-R9A R41A* upon Rpb10 overexpression whereas the rescue was not observed in *hub1-1* upon Rpb10 overexpression. Also, it is likely that the rescue of phenotype observed in the growth assay probably corresponds to the rescue of splicing defects. Thus, the rescue of Hub1 mutants by RNA polymerase subunit Rpb10 suggests a possible link between Hub1, transcription and splicing. However, the mechanism of how Rpb10 rescues Hub1 mutants has to be addressed by further experiments.

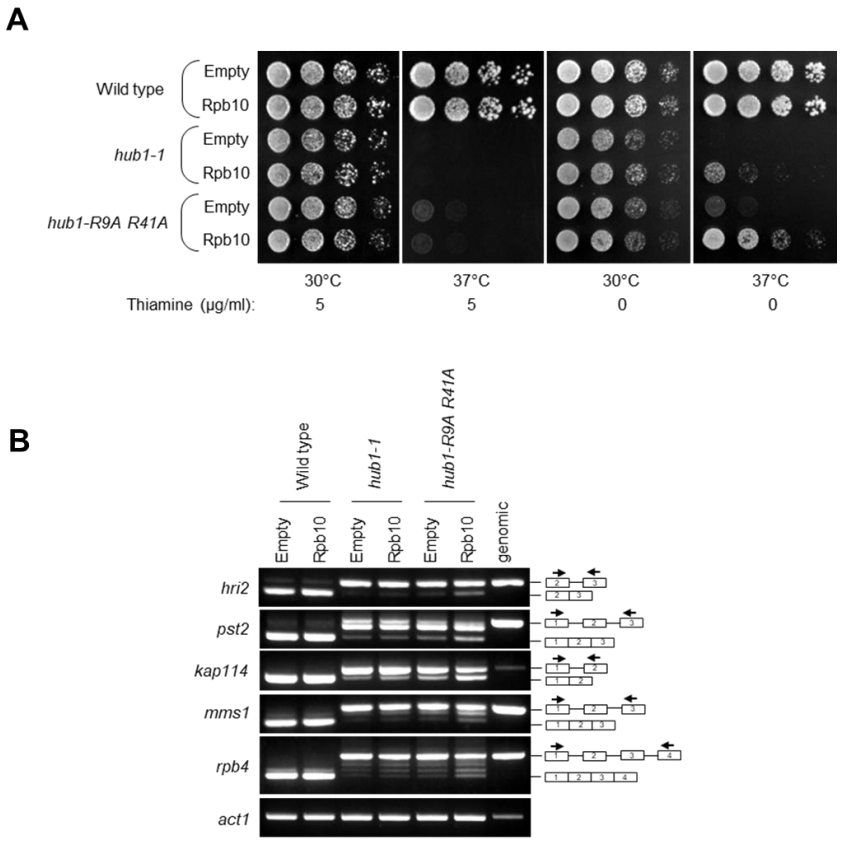

Figure 3.4: Rescue of temperature sensitivity and splicing defects in Hub1 mutants by Rpb10 overexpression

A) Overexpression of Rpb10 in WT, *hub1-1* and *hub1-R9A R41A*. Fivefold serial dilution spot assay shows the rescue of temperature-sensitivity of *hub1-1* and *hub1-R9A R41A* at 37°C upon expression of Rpb10. The spot assay was done on indicated agar plates and incubated at 30°C and 37°C.

B) Semi-quantitative RT-PCR shows a mild rescue of splicing defects for the chosen targets upon Rpb10 overexpression in *hub1-R9A R41A*. 37°C temperature shift for 1 hour was done before harvest. The blocks with the numerical indicate exon numbers and the arrows depict the primers used in this assay.

3.5 Hub1 modifies the spliceosome selectively

Although it is known that Hub1 plays a role in pre-mRNA splicing, its molecular mechanism is not yet known. To gain further insights into the molecular mechanism, a spliceosomal pull-down was done in WT and *hub1-R9A R41A* using 6-HA tagged Cdc5, a core splicing factor and the samples were then analysed by mass spectrometry. It was observed that the level of few splicing factors among which Prp1 increased by manifold in the spliceosome of *hub1-R9A R41A* compared to WT. Nevertheless, most of the components of the spliceosome remained

unchanged in the mutant. Further experiments need to be done to verify the mass spectrometry data. Thus, it can be inferred that Hub1 selectively modulates the composition of the spliceosome.

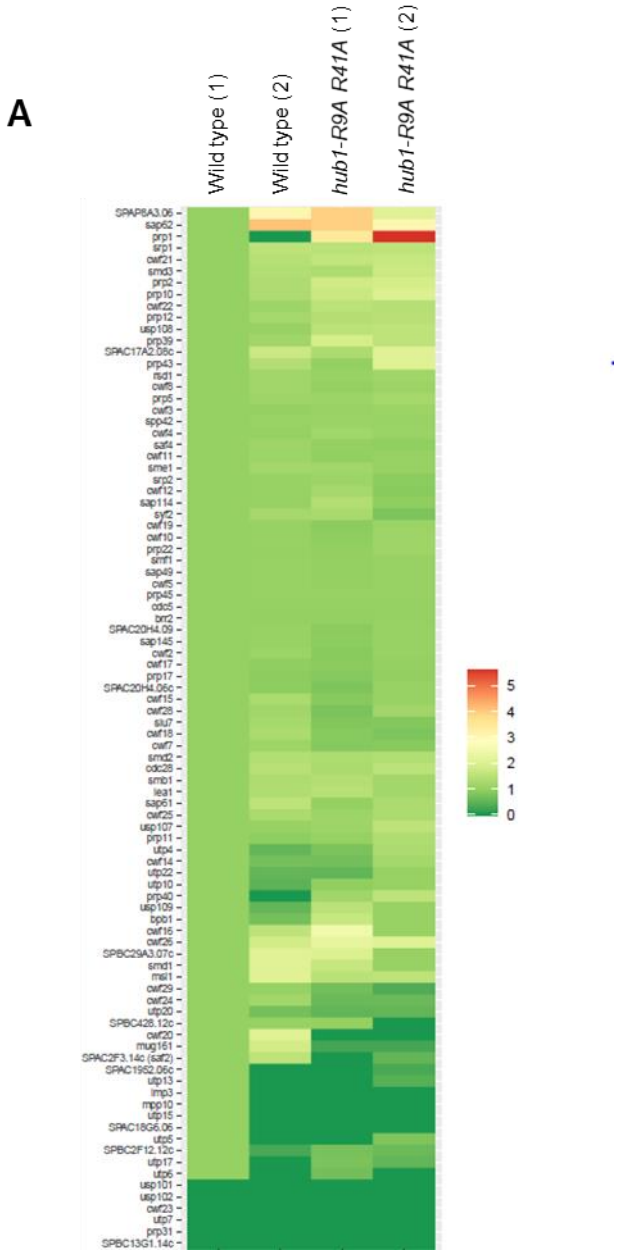

Figure 3.5: Selective modification of spliceosome by Hub1

A) Heatmap shows the normalized values of number of unique peptides obtained for each protein in mass spectrometry of Cdc5-6HA tagged immunoprecipitated complexes in WT and *hub1-R9A R41A* (in duplicates). The heatmap shows values only for spliceosomal proteins. The number of unique peptides obtained for each spliceosomal protein has been normalized with the value obtained for Cdc5 and further normalized with respect to its own value in WT (1). 37°C temperature shift for 3 hours was done before harvest (Cdc5-6HA pulldown was performed along with Balashankar R).

Chapter 4

Discussion and conclusion

The unconventional ubiquitin-like protein Hub1 is known to play a role in alternative splicing through the usage of non-canonical splice site in *S. cerevisiae* and it also promotes efficient splicing by stimulating the RNA helicase Prp5 (Mishra et al., 2011; Karaduman et al., 2017). In *S. pombe*, the novel R9R41 surface of Hub1 is required for cell growth and splicing. The comparative analysis with other surface mutants of Hub1 clearly indicates the importance of Hub1 as growth defects and strong splicing defects are observed in *hub1-R9A R41A*.

The bioinformatic analysis done by Dr. Kiran Kumar in collaboration with Dr. Arashdeep to identify common characteristic feature of Hub1 dependent targets shows a negative correlation between synthesis time of Hub1 dependent transcripts and the splicing defects. Further validation of the bioinformatic analysis done in this study clearly indicates that the transcripts which are synthesised faster are dependent on Hub1 for splicing. Interestingly, the negative genetic interaction observed between Hub1 and Spt4 suggests the possibility that both of them function in non-linear pathways. Further, overexpression of Rpb10, a subunit of RNA pol I, II, III rescues the temperature-sensitivity and splicing defects of Hub1 mutants. It is possible that Rpb10 overexpression slows down the RNA pol thereby causing a rescue of Hub1 mutants. It would be interesting to address this possibility by further experiments. All these observations suggest the possibility of Hub1 playing a role in transcription. It is known that the transcription machinery and the spliceosome are functionally coupled to each other as some of their components play a role in both the functions. Thus, Hub1 could be one of the players in linking transcription and splicing. Identifying novel interacting partners of Hub1 would give better insights into its link between transcription and splicing.

The spliceosome being a highly dynamic complex undergoes extensive conformational changes at every stage of its assembly. In *S. cerevisiae*, an enrichment of certain proteins of U1 and U2 snRNPs was observed in Hub1 deficient cells although most of the components of the spliceosome remained unchanged (Mishra et al., 2011). Similarly in *S. pombe*, almost no change in composition was observed for most of the spliceosomal components except an increased accumulation of Prp1 in *hub1-R9A R41A*. Prp1 is a component of U4/U6.U5 tri-snRNP, associates with pre-catalytic spliceosomal complex and helps in maintaining the integrity of this complex (Lützelberger et al., 2009). It is possible that the accumulation of Prp1

stalls the spliceosome and prevents its further transition. Thus, it is likely that Hub1 functions in displacing factors like Prp1 from the spliceosome to allow further transition.

Since Hub1 might be involved in regulating the dynamic rearrangements and transition, it is possible that transcripts that are synthesised faster require faster transition dynamics of the spliceosome which demands for regulatory proteins like Hub1. This can explain why Hub1 dependent targets are synthesised faster. However, further experiments have to be done to confirm this.

Appendix

Table S1 - Strain list

Strain ID	Relevant genotype	Method	Reference
SP1	h- *ade6-M216, leu1, ura4-D18*	Obtained from Tanaka's lab	
SP10	h- PEM2 *hub1-I42S::Nat-NT2*		This study
SP42	h+ *cdc5–6HA::KanMX4*		This study by Prashant Pandit
SP202	h- PEM2 *hub1-D22A::Nat-NT2*		This study by Dr. Kiran Kumar
SP201	h- PEM2 *hub1-H63L::Nat-NT2*		This study by Dr. Kiran Kumar
SP222	h- PEM2 *hub1-R9AR41A::Nat-NT2*		This study by Dr. Kiran Kumar
SP243	h- PEM2 *hub1-D22A H63L::Nat-NT2*		This study by Dr. Kiran Kumar
SP300	PEM2 *hub1-R9AR41A::Nat-NT2 cdc5–6HA::KanMX4*	Mating and dissection of SP222 and SP297	This study
SP304	h+ *Δspt4::KanMX6*	Cassette based deletion of *spt4* with selection marker using D583	This study
SP307	PEM2 *hub1-I42S::Nat-NT2 Δspt4::KanMX6*	Mating and dissection of SP10 and SP304	This study
SP308	PEM2 *hub1-R9AR41A::Nat-NT2 Δspt4::KanMX6*	Mating and dissection of SP222 and SP304	This study

Table S2 - Plasmid list

Plasmid ID	Plasmid	Method	Reference
D196	*pREP81X-Rpb10*		This study by Dr. Kiran Kumar

D501	*pmug37-3MYC–gmug37*		This study by Dr. Kiran Kumar
D581	*pREP81X-3MYC–gmug37*	Genomic *mug37* with 3MYC tag at N-terminus in pREP81X	This study
D583	*pFA6a.KanMX6* clone for *S. pombe spt4* deletion	Promoter and terminator of *S. pombe spt4* in *pFA6a.KanMX6* for chromosomal replacement	This study

Table S3 - Primer list for RT-PCR

Primer ID	Primer name	Primer sequence (5'-3')
SKM_PR 13	*act1* F	CCCCTAGAGCTGTATTCCC
SKM_PR 13	*act1* R	CCAGTGGTACGACCAGAGG
SKM_PR1235	*gnd1* F	CCGTACAACTTCCAGAGTTGACGAG
SKM_PR1236	*gnd1* R	CAAATTCCTCAAGGGAGTGAGCACC
SKM_PR1290	MYC-F	AGCTGTCGACCGAGATGGGTGAACAAAAG
SKM_PR1356	*kap114* F	ATGGTTGAAAGCAAAATCATTAAGC
SKM_PR1357	*kap114* R	GCAAGTTGAGGTGCAATAATAAAG
SKM_PR1972	*mug37* R	CTTCAAAGTAGTAGAGGATGAC
SKM_PR2046	*rpl2501* F	ACATGGATCCATGAGCGTTGCTAAAGCCAAAG
SKM_PR2048	*rpl2501* R	GGCTTTAAGATGGACATGGAA
SKM_PR2280	*hri2* F	GCGGATGCTTTTAACTGCTTTG
SKM_PR2281	*hri2* R	TCAAATACATTGGTGGGATCGG
SKM_PR2282	*mms1* F	GCAACTCCCAAGAGATTACTTG
SKM_PR2283	*mms1* R	GCGAAGTTCTATAGCATTGCTG
SKM_PR2284	*pcf11* F	CATACTCTTACTTATATCGCGC
SKM_PR2285	*pcf11* R	GGTGTATGCACTCATAAATGTC
SKM_PR2594	*pst2* F	ATGGAACAAACACTAGCGATATTAA
SKM_PR2595	*pst2* R	GAAGTTGGCACCGCTATTCG
SKM_PR2604	*rpb4* F	GCCGAGGGCTATTTTTGAGG
SKM_PR2605	*rpb4* R	CGCAAAGTGGAAAGCTCATC

SKM_PR2672	*sod2* F	CTAAGGCTACTCTTCCCCC
SKM_PR2673	*sod2* R	GGTGATGCTTGTCATGATG
SKM_PR2908	*vta1* F	GAGACTAGTGCAAAGGCGTATG
SKM_PR2909	*vta1* R	GGCTTCTTGCGTAGGTAGGG
SKM_PR2918	*ppk18* F	CGCAATTCCAACGAGGATAAC
SKM_PR2919	*ppk18* R	GGGCAGCTTCGAAATCACGG
SKM_PR2920	*rps1901* F	TGTTAAGGATGTGGACGCTC
SKM_PR2921	*rps1901* R	TAGATGTGGCGGGCAATGGC

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