Function and regulation of a deubiquitinating enzyme module at the Golgi

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A thesis submitted for the partial fulfillment
of the degree of

Doctor of Philosophy

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September, 2023

Dedicated to my family

DECLARATION

The work presented in this thesis titled ‘Function and regulation of a deubiquitinating enzyme module at the Golgi’ 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 universityor 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.

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

Date: Dr. Shravan Kumar Mishra

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ACKNOWLEDGEMENT

I would take this opportunity to express my sincere gratitude to my family, friends, mentors, colleagues, and well-wishers who have played a very important role in my Ph.D. journey.

I am extremely grateful to my Ph.D. supervisor Dr. Shravan Kumar Mishra for his constant support, guidance, and encouragement that led to the successful completion of my PhD journey. His patience and curiosity toward science have and is continuing to inspire me immensely. I particularly thank him for motivating me and letting me try out new ideas in the lab. This has helped to keep my passion and love for science intact. If I have to restart my Ph.D., I would still like to do it under his guidance.

I also thank my doctoral committee members, Dr. Mahak Sharma and Dr. Sudip Mandal for their critical comments and suggestion on the work.

I express my immense gratitude to all faculties and students of the Department of Biological Sciences, IISER Mohali, for providing me access to departmental facilities and helping me perform many of my experiments.

I am thankful to all non-teaching staff members, store and purchase sections, account sections, hostel caretakers, mess and canteen people, cleaning staff, and administration for their help during my stay in IISER Mohali.

I would like to thank our collaborators, Dr. Monika Sharma, Dr. Arashdeep Singh, and Dr. Sittinan Chanarat who have lent their support in the completion of different projects.

I am immensely grateful to Sumanjit, Karan, Sandra, and Pravar for being my teachers when I joined the lab. Prashanth, Poonam, Kiran, and Poulami were excellent seniors who made me feel comfortable during my initial stay in the lab. I would like to thank all the other past and present members of the lab, Balashankar, Bhavya, Nivedha, Aiswarya, Amjad, Sugata, Ankita, Khalid, Kanika, Ashis, Onika, Sukanya, Abhilasha, Srishti, Swaroopa, Arpita, Ompal, and Nishaant for making my stay in the lab an enjoyable experience. I would like to thank Vidya for helping in the lab and teaching me Hindi.

My interest in biology was identified and cultivated by my 10th-standard biology teacher, Mrs, Lakshmipriya. She is the reason why I am pursuing research in biology. I am extremely grateful to her.

During my stay at Anna University, Dr. Tamilselvan served as a mentor and helped me understand the experimental part of science. I would not have joined IISER Mohali if it were not for him. I thank him for supporting and believing in me. Dr. Gautam Pennathur also supported and encouraged me to pursue Ph.D. I thank him for his encouragement.

Any of this would not have been possible without the support of my parents. They had complete faith in my decision and gave me the freedom to pursue my goals. Their love and support gave me the strength to sail through my life. I also thank my sister, Dharshini for her love and support without expecting anything in return.

I shared the majority of my Ph.D. journey with Anupa. I am extremely lucky to have found my friend, my colleague, and my life partner in her. She made my life both in the lab and outside the lab peaceful. I am eternally grateful to her for agreeing to be my partner in crime throughout my life.

Last but least, I would to thank IISER Mohali and India Alliance Wellcome DBT for funding my stay as well as projects in the lab.

All may not be mentioned, but no one is forgotten.

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ABBREVIATIONS

ºC	Degree Celsius
%	Percentage
A	Adenine
ATP	Adenosine triphosphate
BP	Branch point
cDNA	Complementary DNA
Co-IP	Co-immunoprecipitation
Cys	Cysteine
DNA	Deoxyribonucleic Acid
ESE	Exonic splicing enhancer
ESS	Exonic splicing silencer
FL	Full-length
G	Glycine
H3K9	Histone 3 lysine 9
HDAC	Histone deacetylase
hnRNA	heterogeneous nuclear RNAs
Ile	Isoleucine
IR	Intron-retention
ISE	Intronic splicing enhancer
ISS	Intronic splicing silencer
Lys	Lysine
Met	Methionine
Mg	Magnesium
mM	milli Molar
ns	No signal
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
PPi	Pyrophosphate
PPT	Polypyrimidine tract
Pre-mRNA	Precursor messenger RNA
Prp	Pre-mRNA processing protein
RBP	RNA binding protein
RNA	Ribonucleic Acid
RNAPII	RNA polymerase II

RNP	Ribonucleoprotein
RT-PCR	Reverse transcriptase polymerase chain reaction
Ser	Serine
SnRNAs	Small-nuclear RNA
SnRNPs	Small-nuclear ribonucleoprotein
ss	Splice site
Trp	Tryptophan
UBL	Ubiquitin-like modifier
UTR	Untranslated regions
w.r.t	With respect to
WB	Western blot
wt	Wild-type

SYNOPSIS

FUNCTION AND REGULATION OF A DEUBIQUITINATING ENZYME MODULE AT THE GOLGI

INTRODUCTION

Eukaryotic genes contain coding exons which are largely interspersed by non-coding sequences termed as introns. They are defined by their splicing signals: the 5'-splice site (5'ss), the branchpoint (BP) and the 3'-splice site (3'ss). The splicing of precursor mRNAs into protein-coding mRNAs requires the removal of introns that is mediated by the large ribonucleoprotein complex called the spliceosome. Spliceosome recognizes the introns by their splicing signals and excise them (Matera and Wang 2014). Introns are degenerate, differ in the sequences of their splicing signals, distance between the splicing signals, and location in the precursor mRNA (William Roy and Gilbert 2006). This degeneracy leads to alternative splicing which is crucial for the functioning of intron-rich eukaryotes. To accommodate the splicing of different introns, splicing regulators that include RNA binding proteins, RNA and protein modifiers and ubiquitin and ubiquitin-like proteins (UBL) modify the spliceosome(Y. Lee and Rio 2015).

Sde2 is one such ubiquitin-fold containing splicing regulator in *Schizosaccharomyces pombe.* It is synthesized as an inactive precursor harboring an N-terminal UBL domain ($Sde2_{UBL}$) and a C-terminal domain (^{K}Sde2-C) separated by the invariant GG–K motif. Two deubiquitinating enzymes, Ubp5 and Ubp15, process Sde2 at the GG–K motif to form the active ^{K}Sde2 with free lysine. The ^{K}Sde2 enters the spliceosome and is required for the excision of selected introns in a subset of genes (Thakran et al. 2018). One of targets of Sde2 is the intron-3 of *ftp105* gene. Ftp105 localizes Ubp5 to Golgi (Kouranti et al. 2010). However, the question remains why only a subset of introns require Sde2 for its proper splicing and what is the physiological significance of Sde2-mediated splicing regulation.

Golgi apparatus is the sorting station of the cell that sorts various proteins into the extracellular environments or into the endomembrane organelles. Accurate sorting of proteins is important for the functioning of the cells (Guo, Sirkis, and Schekman 2014). Ubiquitination has been shown to act as an important signal for the protein quality control at the Golgi (Schwabl and Teis 2022).

Ubiquitination is a reversible modification carried by the concerted action of three enzymes: E1 activating enzyme, E2 conjugating enzymes, and E3 ubiquitin ligase. The specificity of the substrate is determined by the E3 ligase. Deubiquitinating enzymes (DUBs) are the proteases that remove the ubiquitin marks from the proteins (Pickart 2001). Although several E3 ligases have been identified and characterized at the Golgi, the role of DUBs is not well explored (Schwabl and Teis 2022). Out of the 20 DUBs in *S. pombe*, Ubp5 is the only known DUB to localize to Golgi. However, the significance of the localization of Ubp5 at Golgi is not known.

The human homolog of Ubp5, USP7, plays an important role in diverse cellular processes including DNA damage response, transcription, immune response to name a few. USP7 overexpression leads to cancer as it decreases the level of the tumour suppressor p53 (Pozhidaeva and Bezsonova 2019). The role of USP7 in sorting cargoes at the Golgi is not known. In *Caenorhabditis elegans* HID-1, the homolog of Ftp105, was identified in a screen for mutants showing constitutive Dauer formation at 27ºC. HID-1 acts in the insulin branch of Dauer formation (Ailion and Thomas 2003). HID-1 was shown to play a role in neuropeptide sorting and secretion by controlling the formation of Large Dense Core Vesicles (LDCVs) (Mesa et al. 2011; Yu et al. 2011). LDCVs are specialized vesicles that stores cargoes such as peptide hormones or neuropeptides in secretory cells (Tooze and Huttner 1990). In pancreatic cells, the loss of HID-1 leads to defective insulin secretion (Du et al. 2016). In rat endocrine cells, HID-1 regulates the sorting of peptide hormones by controlling trans-Golgi network (TGN) acidification (Hummer et al. 2017). Interestingly, HID-1 is a peripheral membrane protein localized primarily in medial and trans Golgi network through N-terminal myristoylation (L. Wang et al. 2011).

Although much is known about the function of HID-1/Ftp105 in vesicular trafficking, the significance of its interaction with Ubp5 is not clear

OBJECTIVES

1) Why only a subset of introns requires Sde2 for its proper splicing and what is the physiological significance of intron-specific splicing?

2) What is the significance of the Golgi-localized deubiquitinating enzyme Ubp5 and its partner Ftp105?

OUTCOME OF THE STUDY

1) Mechanism and physiological significance of intron-specific splicing

Ubiquitin-like processing of Sde2 is essential for its splicing activity

To address the question of intron-specificity of Sde2, we made splicing reporters with the *S. pombe ura4* split with a Sde2-dependent intron 2 of Rap1. We tested the reporters' ability to complement a *ura4* auxotrophic *S. pombe* strain. Functional *ura4* mRNA and protein were expected only after accurate splicing of the pre-mRNAs that would allow the cells to grow on plates lacking uracil (−URA), but not on uracil-counter selection plates containing 5'-fluoroorotic acid (+FOA). Using the *rap1-i2* reporter, we showed that the ubiquitin-like processing of the precursor Sde2 and the N-terminal lysine of ^{K}Sde2-C is essential for the intron-specific splicing activity of Sde2. As a result, the processing-defective Sde2, *S. pombe* deleted of its processing enzymes Ubp5 and Ubp15, and the processed Sde2-C mutated in its N-terminal lysine showed splicing defects similar to the Sde2-deletion strain.

Sde2-dependent introns have longer distance between branch point (BP) and 3' splice site (3'ss)

To identify common features of Sde2-dependent introns, we did a bioinformatic analysis and found that Sde2-dependent introns had a longer distance between branch point (BP) and 3' splice site (3'ss) than other introns in *S. pombe.* To test the significance of a longer BP-3'ss, I made a *ura4* reporter with intron-3 of *ftp105* (Sde2-dependent intron). The distance of BP-3'ss of this intron is 24 nt. When we reduced the distance to 12 nt, we found that the intron gets spliced even in the absence of Sde2. Thus, introns containing longer distance between BP-3'ss are dependent on Sde2 for its efficient splicing.

Selection of BP-distant 3'ss is not a dominant phenomenon

We then checked whether Sde2 could promote the splicing of BP-distant 3'ss even in the presence of BP-near 3'ss. I made a reporter with two competing 3'ss in a Sde2-independent intron. I found that cells prefer the usage of BP-near 3'ss even in the presence of Sde2.

Intron-specific splicing control the expression of RNA interference (RNAi) and heterochromatin factors

In our lab, we identified two more intron-specific factors, Cay1 and Tls1. When we looked into the targets of Sde2, we found that many of them are involved in the assembly of heterochromatin. Also, the deletion of *sde2*, *cay1* and *tls1* show defects in heterochromatin and telomeric silencing. We found that the proteins levels of some of key heterochromatin factors like Rap1, Dsh1, Set3 and Hif2 were reduced in the absence of the intron-specific splicing factors. We also showed that the reduced protein level is due to defective splicing of these heterochromatin factors.

2) Function of the Golgi-localized deubiquitinating enzyme, Ubp5, and its partner Ftp105

Ftp105 and Ubp5 function at the Golgi

I observed that Ubp5 is localized at Golgi. In the absence of Ftp105, Ubp5 showed a diffused localization in the cell. By microscopic analysis, I showed that Ftp105 localizes to Golgi through an N-terminal myristoylation modification. Through genetic interactions and growth assays, I observed that Ubp5 is epistatic over Ftp105 and they function together in the same pathway.

The localization of a high affinity glucose transporter, Ght5 is dependent on Ftp105-Ubp5 complex

To identify the substrates of Ubp5, I performed mass spectrometric analysis to identify the ubiquitinated proteins enriched in the absence of Ftp105 and Ubp5. Interestingly, I found many plasma membrane transporters as the substrates of Ftp105 and Ubp5. One such substrate was Ght5, a high affinity glucose transporter. I found that Ght5 is mislocalized and degraded in the vacuole in glucose-limiting conditions in the absence of Ftp105 and Ubp5. This localization defects possibly led to growth defects of these mutants in glucose-starved conditions. Through genetic analysis, we found that Ght5 may directly get sorted from the Golgi to vacuole in the absence of the Ftp105-Ubp5 complex.

Identification of E2 conjugating enzyme, E3 ligase and arrestin-related trafficking adaptor that sort Ght5 to vacuole

To identify the E3 ligase that ubiquitinates Ght5, we performed a genetic screen. The idea was that DUBs' role on a substrate would become redundant in the absence of the E3 ligase that ubiquitinates it. E3 ligases were co-deleted in the background of *ftp105* deletion and the double mutants were tested for growth rescue. Using this strategy, I identified Pub1 as one of the E3 ligases which sorts Ght5 into the vacuole. The localization defect of Ght5 observed in the absence of Ftp105 was rescued when Pub1 or its cognate E2 conjugating enzyme, Ubc4, was inactivated. Rsp5, the homolog of Pub1 in *Saccharomyces cerevisiae* binds to group of adaptor proteins called arrestin-related trafficking adaptors to downregulate transporters. Using a similar genetic screen and localization studies, we found that Rod1 and Aly3 are the arrestins involved in the sorting of Ght5 to the vacuole.

CONCLUSION

In my doctoral study, I found that the introns containing a longer distance between branch point and 3' splice site requires the intron-specific splicing factor, Sde2, for its efficient splicing. I also found that the intron-specific splicing factors regulate heterochromatin silencing by ensuring the proper splicing of key heterochromatin proteins. My study shed light on the role of specialized splicing factors in promoting the splicing of suboptimal introns containing BP-distant 3'ss.

In the second part of my doctoral study, I found that the Sde2-activating DUB Ubp5 is Golgi-localized and together with its interacting partner Ftp105, promote the proper localization of a glucose transporter Ght5 to the plasma membrane in glucose-limiting conditions by counteracting the activity of the E3 ligase Pub1 and arrestin-related trafficking adaptors, Rod1 and Aly3. This is one of the first report that details the role of Golgi-localized DUB module in sorting of cargoes from Golgi to plasma membrane.

In conclusion, my thesis work revealed the mechanism of Sde2-dependent splicing of Ftp105 which in turn works with the deubiquitinating enzyme Ubp5, in regulating the proper trafficking of a sugar transporter.

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CHAPTER 1: Mechanism and significance of splicing of introns containing branchpoint-distant 3’ splice sites

1.1 ABSTRACT

Eukaryotic genes contain coding exons which are largely interspersed by non-coding introns. The splicing of precursor mRNAs into protein-coding mRNAs by the removal of introns is mediated by the large ribonucleoprotein complex called the spliceosome. Although the role of core splicing factors is well-characterized, the significance of specialized splicing regulators is not well understood. Sde2 is one such highly conserved regulator. It is a ubiquitin-fold-activated splicing regulator required for the efficient splicing of selected introns in a subset of genes in *Schizosaccharomyces pombe*.

In the first part, we wanted to identify why only selected pre-mRNAs required Sde2 for its efficient splicing. By bioinformatic analysis and monitoring splicing-specific *ura4* reporters, we identified that introns with longer spacing between branchpoint and 3’ss splice site (for example, *ftp105* intron-3) required Sde2 for its proper splicing. We also found that ubiquitin-like processing is required for the proper activity of Sde2. Additional intron-specific splicing factors, Cay1 and Tls1 along with Sde2, regulate heterochromatin formation and telomeric silencing by ensuring the proper splicing of key heterochromatin factors. Thus, splicing of suboptimal introns with longer spacing between branchpoint and 3’ss required intron-specific splicing factors for its efficient splicing and this in turn regulates the formation of heterochromatin.

1.2 INTRODUCTION

1.2.1 RNA splicing

Post transcriptional regulation is very important to gene expression and one of the key modifications is RNA splicing. Pre-mRNA splicing was first discovered in adenovirus and is found only in eukaryotes. Genes contain coding exons that are interspaced by sequences called introns (Figure 1.2.1). Introns are marked by their splicing signals: the 5'-splice site (5'ss), the branchpoint (BP) and the 3'-splice site (3'ss). These signals are recognized by a megadalton RNA-protein complex called spliceosome (Burge et al. 1993).

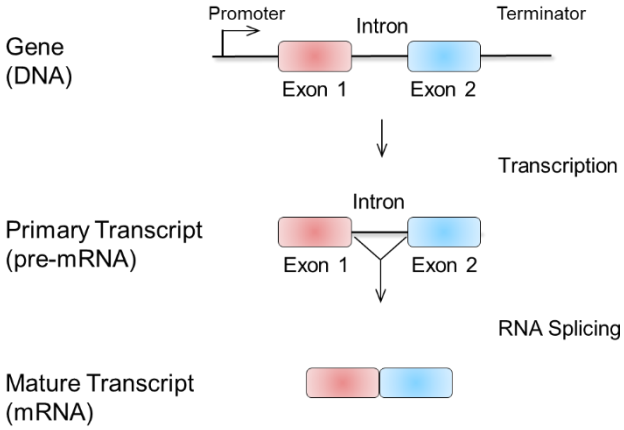


Figure 1.2.1: RNA splicing.

Introns are removed and exons are ligated in a process called RNA splicing. This process leads to the generation of translatable mRNA.

The pre-mRNA splicing involves two transesterification reactions which are catalyzed by the spliceosome. In the first reaction, the phosphodiester bond at the 5' splice site (5'ss) is attacked by the 2'-hydroxyl of an adenosine of the branch point sequence (BP) in the intron. It produces a free 5' exon and an intron-lariat-containing exon. In the second transesterification reaction, the 3'+-hydroxyl of the free exon attacks the phosphodiester bond at the 3'ss of the lariat-containing exon, leading to exon ligation and removal of the lariat intron (Figure 1.2.2). The splicing reaction is facilitated by two divalent metal ions. The RNA components of the spliceosome position these ions, hence the catalysis is carried out by RNA (Steitz and Steitz 1993)(Fica et al. 2013).

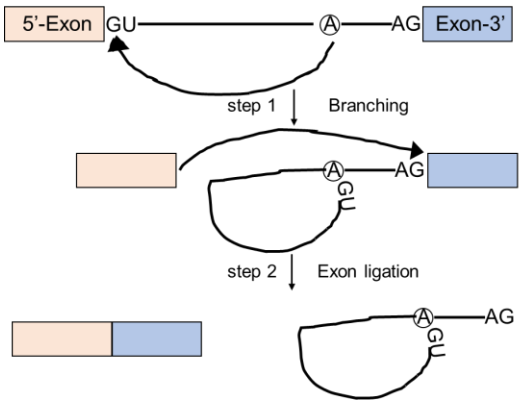


Figure 1.2.2: Two steps of pre-mRNA splicing.

The two steps of the transesterification reactions lead to the splicing of exons.

1.2.2 Assembly of spliceosome

Spliceosome consists of U1, U2, U4/U6, and U5 snRNAs and several proteins. Spliceosome is assembled in a step-by-step manner on the pre-mRNAs (Figure 1.2.3) (Will and Lührmann 2011). Assembly of spliceosomes into a catalytically competent conformation requires major structural rearrangements which are performed majorly by eight DEXD/H-box RNA helicases (Figure 1.2.4) (Cordin and Beggs 2013). Substrate recognition and catalysis are performed by the snRNAs.

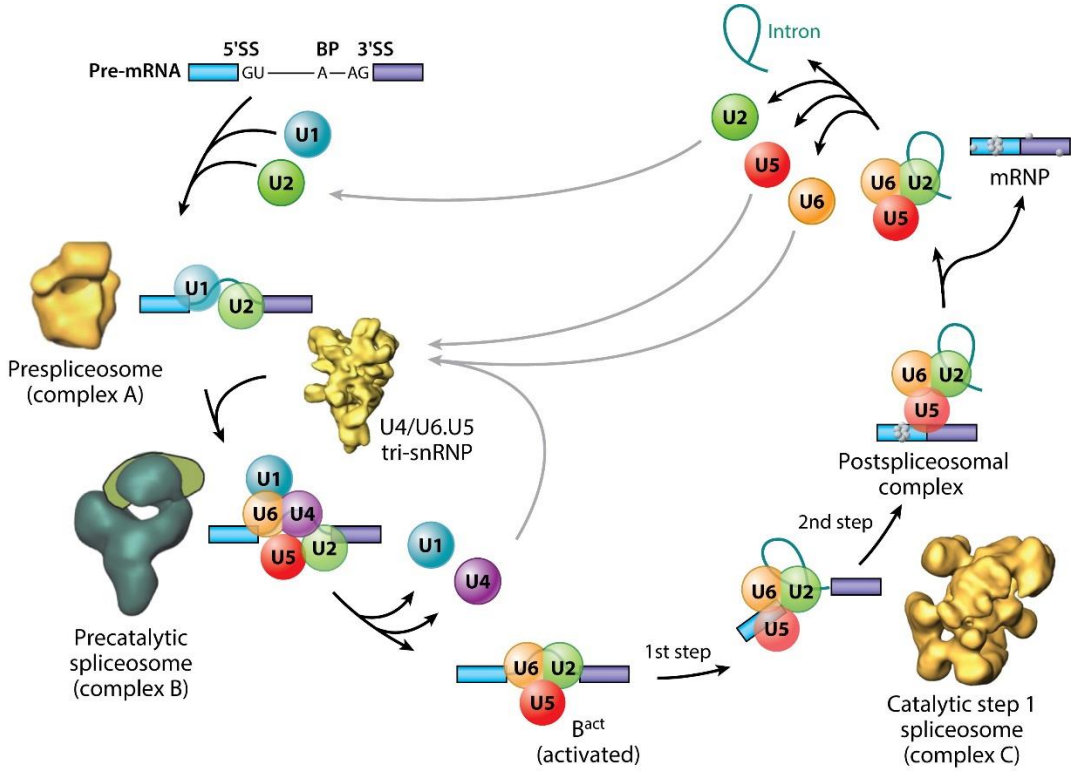


Figure 1.2.3: Assembly and disassembly of spliceosome with known structures.

Taken from the reference (Lee and Rio, 2015).

In the initial steps, the U1 snRNP and the U2 snRNP recognizes the 5'ss and the BP sequence respectively (Plaschka et al. 2018). This is the commitment step of the splicing which is regulated by trans-acting proteins and cis-regulatory elements of the RNA. As this step determines the splice-site selection, it plays an important role in alternative splicing (Ule and Blencowe 2019). The U1 snRNA binds to the 5'ss weakly and this binding is stabilized by U1-associated proteins. The U2 snRNP binds the BP sequence leading to the formation of prespliceosome or the A complex. The U2 snRNA along with its associated proteins SF3b and SF3a associate with the BP sequence to form the branch helix. In the next step, the largest pre-assembled complex of the spliceosome, the U4/U6.U5 tri-snRNP, enters the spliceosome. The U6 snRNA forms the catalytic centre of the spliceosome which is kept in an inactive conformation via its binding with U4 snRNA (Nguyen et al. 2015). Prp8, the largest and the highly conserved protein of the spliceosome, enters with the tri-snRNP complex. Prp8 crosslinks with all the three splice sites: the 5'ss, the BP and the 3'ss. Hence, it is considered to be the heart of the spliceosome (Grainger and Beggs 2005). The binding of tri-snRNP with the A complex leads to the formation of pre-B complex. The pre-B complex is held together by the interaction with U2 and U6 snRNAs. Then, the RNA helicase Prp28 removes the U1 snRNA bound to 5'ss and transfers the 5'ss to

base pair with the conserved ACAGA box of U6 snRNA (Staley and Guthrie 1999). This leads to the release of the U1 snRNP and the formation of the B complex. The U5 snRNA interacts with the 3'-terminal nucleotides of the 5'exon. This interaction is important for the catalytic reactions (Turner et al. 2004). The helicase Brr2 then unwinds the U4 snRNA binding with the U6 snRNA (Raghunathan and Guthrie 1998). The U6 snRNA then pairs with the U2 snRNA to form the catalytic centre (Madhani and Guthrie 1992). This leads to the formation of the B^{act} complex. During the conversion of the B complex to the B^{act} complex, the largest exchange of proteins happens. The U4 snRNP associated proteins leave and the NTR/NTC complex proteins arrive. The catalytic centre of the U6 snRNA is stabilized by the NTR/NTC proteins (Rauhut et al. 2016). Even though the catalytic core is formed, the 1^{st} step does not happen because the BP adenosine sequence is bound by HSH155 protein of the SF3B complex (Rauhut et al. 2016). And the 5'ss is masked by the SF3a protein Prp11 and Cwc24 (Nan-ying Wu, Chung, and Cheng 2017). The Prp2 helicase then relieves the inhibition on the BP adenosine and 5'ss to convert the B^{act} complex to the B* complex (Cordin, Hahn, and Beggs 2012). In the B* complex, the first step of splicing takes place leading the formation of the C complex. The active site for the second step remains the same as the first step. For the exon ligation to happen, the branch helix has to be displaced. The helicase activity of Prp16 displaces the branch helix (Schwer and Guthrie 1992). This remodeling leads to the exon-ligation conformation which is the C* complex. In the C* complex, the 3'ss is docked with the help of Slu7 and Prp18 (Semlow et al. 2016). This leads to the exon ligation. Next in the P complex, the Prp22 helicase releases the spliced exons (Company, Arenas, and Abelson 1991). The Intron-lariat spliceosome and the snRNAs are recycled by the activity of Prp43 helicase (Arenas and Abelson 1997).

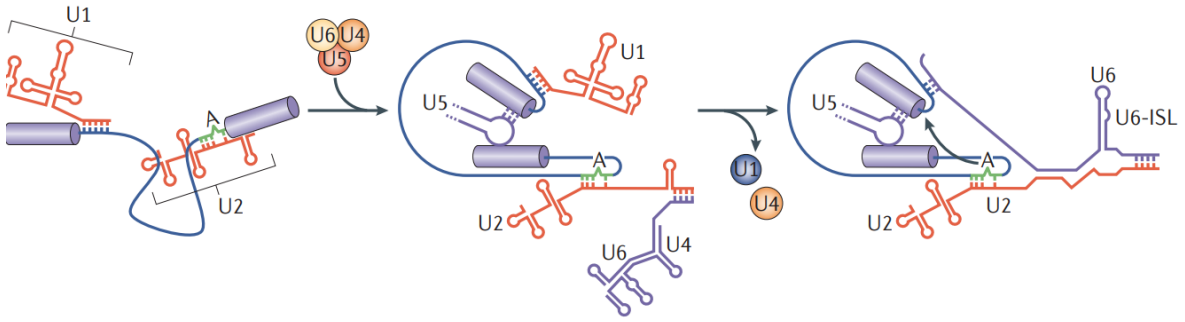


Figure 1.2.4: Rearrangement of RNA-RNA interaction in the spliceosome.

Schematic representing the RNA–RNA interactions in an ordered manner to form the catalytic centre during splicing. A indicates the branch point. The U6-ISL is the intramolecular stem-loop which is important for splicing. Taken from the reference (Matera and Wang 2014).

1.2.3 Exon and intron definition model of splicing

Since the splice sites are degenerate, one of the major challenges faced by the spliceosome is the accurate recognition of the splice sites from other sequences. But it has been shown that the

spliceosome recognizes the splice sites quite efficiently. The question then arises how does the spliceosome define the intronic and exonic regions. There are two models of recognition which has been described.

In vertebrates, the introns are very long and the exons are comparatively shorter (M. Q. Zhang 1998). So, it is easier for the spliceosome to assemble on the exon than the intron. In the exon, U1 and U2 snRNPs interact with each other and define and recognize the exon. This is defined as the exon definition model of splicing (Berget 1995). In the subsequent steps, rearrangements happen in the U1 and U2 snRNPs leading to the intron definition. In lower eukaryotes, the introns are relatively shorter in length and it only follows the intron definition model of splicing (De Conti, Baralle, and Buratti 2013).

1.2.4 Alternative splicing

As discussed previously, the introns can have very degenerate splice sites. This is especially the case in higher eukaryotes. This degeneracy in the splice signals leads to alternative splicing of pre-mRNAs leading to generation of protein isoforms. So, in higher eukaryotes, additional protein factors are required to stabilize the binding of snRNAs to these splice sites. The requirement of additional factors in recognizing these degenerate splice sites can add another layer of spatial and temporal regulation of alternative splicing. It has also been observed that as the complexity of organisms increase, the alternative splicing events for a particular gene also increase with humans being among the highest (Barbosa-Morais et al. 2012). Given the importance of splicing in generating various protein isoforms, mutations either in the introns or the spliceosomal machinery leads to various diseases (Padgett 2012).

Alternative splicing takes place generally in seven ways: i) exon skipping, ii) mutually exclusive exons, iii) alternative donor sites, iv) alternative acceptor sites, v) intron retention, vi) alternative promoter, and vii) alternative polyadenylation sites (Kolathur 2021; Wahl and Lührmann 2015). In humans, the most prevalent mode of alternative splicing event is the exon skipping (Figure 1.2.5).

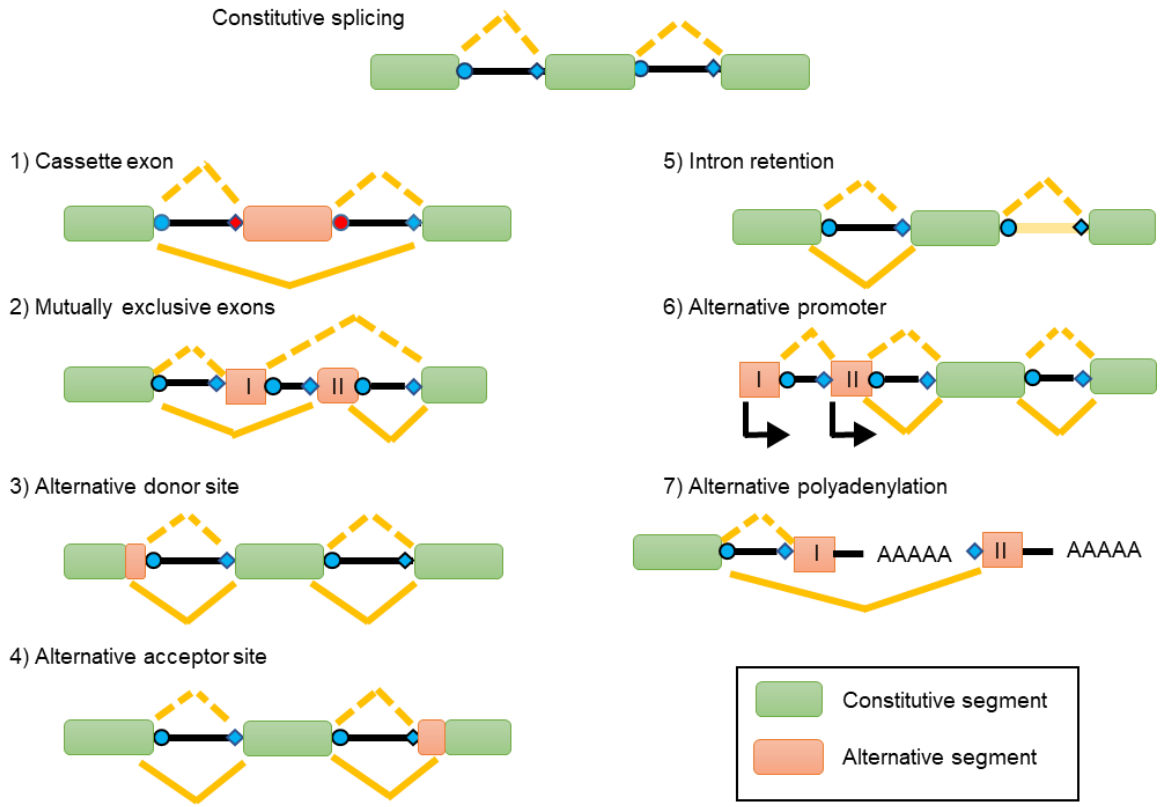


Figure 1.2.5: Types of alternative splicing.

Schematic represents various type of alternative splicing events. Bold lines represent canonical splicing whereas dashed lines represent alternative splicing events. The 5'ss and 3'ss are represented by circle and diamond respectively.

To aid in the proper recognition of introns, additional sequence features other than the core splicing signals are present in the pre-mRNA. These splicing regulatory elements (SREs) are classified as exonic splicing enhancers (ESEs), intronic splicing enhancers (ISEs), exonic splicing silencers (ESSs) and intronic splicing silencers (ISSs). These SREs recruit several RNA-binding auxillary factors (Figure 1.2.6). The RNA-binding factors can either enhance or repress the splicing. These factors act mostly on the early steps of spliceosomal assembly. One of the well-studied examples is the splicing inhibition by the polypyrimidine tract-binding (PTB) protein. As the name suggests, PTB binds to the polypyrimidine tract of mRNA. This bound PTB interacts with the U1 snRNA and prevents the further transition of spliceosome to take place (Sharma et al. 2011). One of the SR proteins, the family of proteins with RS domain (arginine and serine rich), helps the U1 snRNA to bind with the 5'ss (Kohtz et al. 1994). SR proteins generally act as splicing activators that facilitates proper splicing by mostly binding to ESEs. The SR-related proteins SRRM1 and SRRM2 binds to U2 snRNP and promotes the splicing of pre-mRNA from the *Drosophila doublesex* gene (Eldridge et al. 1999). Phosphorylated RS domain of the SR proteins binds double-stranded RNA and stabilizes the binding between snRNAs and splice sites (Shen and Green 2006). hnRNP family of proteins act as the negative regulators of exon recognition by binding to ESS and ISS elements (Dreyfuss, Kim, and Kataoka 2002). The hnRNP L

along with hnRNP A1 promotes the exon skipping of CD45 exon 4. They do so by stabilizing the binding of U1 snRNA to the 5'ss and neighboring exonic sequences. This binding prevents the association of U6 snRNA with the 5'ss, thereby repressing the splicing (Chiou, Shankarling, and Lynch 2013).

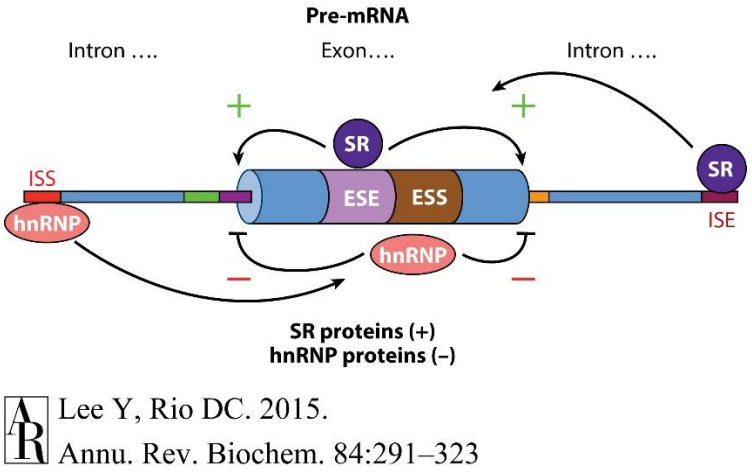


Figure 1.2.6: Regulation of pre-mRNA splicing by cis- and trans-acting elements.

The different splicing regulatory elements (SREs) present in the pre-mRNA and the trans-acting proteins which bind them are indicated. + and – indicates positive and negative regulation of splicing respectively (Lee and Rio, 2015).

RNA secondary structures also play an important role in regulating alternative splicing (Buratti and Baralle 2004). Rbfox splicing factor mediated exon inclusion of ENAH is dependent on conserved long-range RNA-RNA base-pairing interaction (RNA bridge) (Lovci et al. 2013). The 5'ss of exon 10 of *TAU* gene contains a stem-loop structure. Stabilization of the stem-loop structure of exon 10 leads to exon-skipping while destabilization leads to exon-inclusion (Donahue et al. 2006). Trans-acting RNA-RNA base-pairing also influences alternative splicing. The small nucleolar RNA (snoRNA), HBII-52, binds to the silencer region of alternatively spliced exon Vb of the serotonin receptor 5-HT2CR. This leads to the formation of different protein isoforms (Kishore and Stamm 2006).

Splicing has been shown to take place co-transcriptionally in most of the cases (Perales and Bentley 2009). The snRNPs are recruited to the mRNAs during the process of transcription itself (Lacadie and Rosbash 2005). The elongation rate of the RNA polymerase II have been shown to influence alternative splicing (Dujardin et al. 2013, 2014; Ip et al. 2011; de la Mata et al. 2003; Roberts et al. 1998). Recently, it has been shown that changes in chromatin structure also influences alternative splicing. Nucleosomes are known to be concentrated around the exons than the introns (Schwartz, Meshorer, and Ast 2009). Interestingly, exons with weaker splice elements have more stable nucleosome occupancy and exons included in the alternatively spliced product also have more nucleosome occupancy (Tilgner et al. 2009). This indicated the role of nucleosome positioning in

alternative splicing. In addition to nucleosome positioning, histone modifications and mRNA modifications have also been shown to regulate alternative splicing (Luco et al. 2011).

1.2.5 UBLs

Proteins can be modified through conjugation to different proteins and other molecules such as phosphate and acetyl groups. Ubiquitin is one such highly studied modifier. The process of covalent conjugation of ubiquitin to other substrates is mediated by the combined action of three enzymes: the E1 activating enzyme, the E2 conjugating enzyme, and the E3 ubiquitin ligase. Although ubiquitin was initially characterized for its role in proteasomal degradation, it affects a wide variety of processes such as DNA repair, autophagy, endocytosis to name a few (Pickart 2001). It has a compact structure with a β-grasp fold (Vijay-Kumar, Bugg, and Cook 1987). In recent years, several proteins which have a similar a β-grasp fold have been identified. These class of proteins which are similar in sequence as the well as structure to that of ubiquitin are referred as ubiquitin-like proteins (UBLs) (Figure 1.2.7) (Hochstrasser 2009).

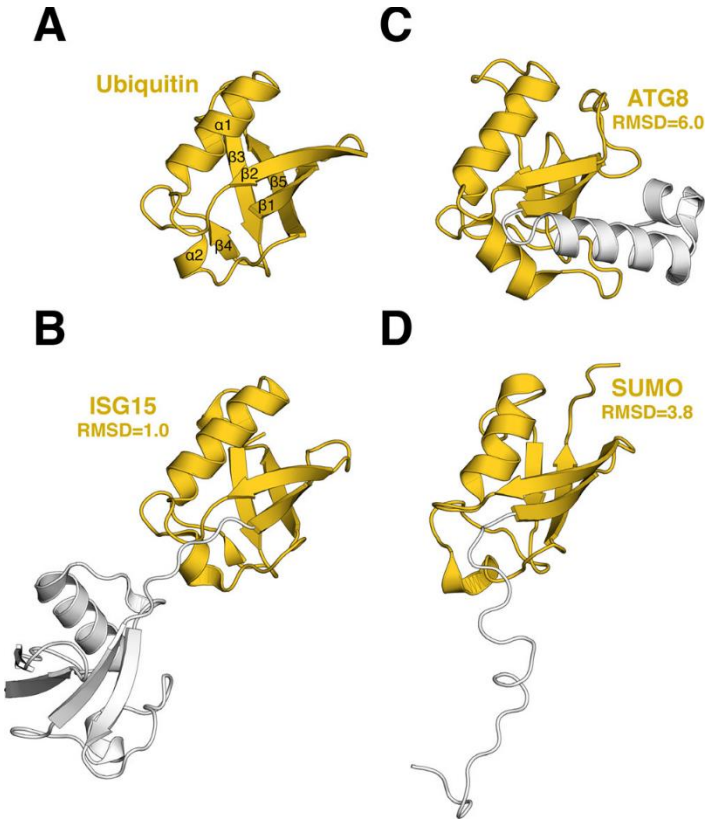


Figure 1.2.7: Structure of different UBLs with the PDB IDs.

A) Ubiquitin structure. B) ISG15 structure. The grey colored area indicates one of β-grasp domain. C) ATG8 structure. The grey colored region indicates the N-terminal extension. D) SUMO structure. The grey colored region indicates N-terminal extension. Root-mean-square deviations (RMSDs) are assessed between Cα of ubiquitin and the Cα of other UBLs. Taken from the reference (Cappadocia and Lima 2018).

Most of the UBLs are also known to get conjugated to proteins similar to ubiquitin (Schulman and Wade Harper 2009). One of the first characterized UBL, ISG15 is stimulated by interferons and covalently modifies other proteins (Haas et al. 1987; Loeb and Haas 1992). It is known to play an important role in enhancing innate immune response (Malakhova and Zhang 2008). After the discovery of ISG15, several UBLs such as SUMO, Nedd8, Atg8, Atg12, Ufm1, Urm1, Hub1 etc., have been characterized and they have been shown to play an important role in diverse cellular pathways (Figure 1.2.8) (van der Veen and Ploegh 2012).

UBL	Function	Substrates	Comments
Ubiquitin	26S proteasomal degradation, chromatin remodelling, immune system regulation, endocytosis/ trafficking	Short-lived proteins, histones, signalling proteins, membrane proteins (ion channels and receptors)	Diversity of Ub function stems from its ability to form polyubiquitin chains using one of its seven lysine residues or N-terminus and also from the recognition of numerous distinct forms of Ub (including monoubiquitin and different Ub polymers).
SUMO	DNA repair, nuclear transport, transcription	TDG, Ran GTPaseactivating protein 1, activators and repressors of transcription	SUMO primarily has nuclear-related functions. Mammals have three active isoforms, two of which have the ability to form polySUMO chains.
NEDD8	Activation of cullin E3 Ub ligases	Cullins	Cullin E3s are the largest class of Ub ligases that regulate key cellular processes, such as the cell cycle.
Atg12	Autophagy	Atg5	Atg12–Atg5 acts as an E3 for the Atg8 pathway.
Atg8	Autophagy	PE	Mammals have Atg8 homologues

Figure 1.2.8: Different UBLs and their known functions.
Adapted from (Taherbhoy, Schulman, and Kaiser 2012).

Pre-mRNA splicing as discussed earlier, is a tightly regulated process (Will and Lührmann 2011). In recent years, it has been found that ubiquitin and other UBLs also regulate the process of splicing (Chanarat and Mishra 2018). Prp8 contains Jab1/MPN domain in its C-terminus with which it interacts non-covalently with ubiquitin (BELLARE et al. 2006). Later, Prp8 was also shown to be ubiquitinated (Bellare et al. 2008). In the same study, it was shown that supplementing a dominant-negative form of ubiquitin-144A reduced the levels of tri-snRNPs (Bellare et al. 2008). Prp19 complex protein ubiquitinates Prp3 which facilitates its interaction with Prp8. The deubiquitinating enzyme USP4 with its partner Sart3 then deubiquitinates Prp3 for the proper maturation of the spliceosome (Song et al. 2010). Thus, the cycle of ubiquitination and deubiquitination controls the proper progression of the assembly of spliceosome. Proteomic analysis revealed that many splicing factors are SUMOylated (Psakhye and Jentsch 2012). Prp3 was shown to be SUMOylated which mediates its interaction with the spliceosome and is required for efficient pre-mRNA splicing (Pozzi et al. 2017).

Hub1 is also a member of the UBL family of proteins (Lüders, Pyrowolakis, and Jentsch 2003; Wilkinson et al. 2004; Yashiroda and Tanaka 2004). It lacks the diglycine motif which is conserved in most of the UBLs and it has not been shown to get conjugated to proteins (Ammon et al. 2014;

Karaduman et al. 2017). Hub1 is a highly conserved protein which has been shown to play an important role in pre-mRNA splicing in *S. cerevisiae*, *S. pombe*, *C. elegans*, and humans (Ammon 2013; Karaduman et al. 2017; Kolathur et al. 2023; Shravan Kumar Mishra et al. 2011). In *S. cerevisiae,* Hub1 binds to Snu66, a component of the U4/U6.U5 tri-snRNP complex. The Hub1-interacting domain (HIND) at the N terminus of Snu66 binds Hub1 and this interaction is essential for Hub1's activity in regulating the usage of introns with weak or non-canonical 5'ss (Shravan Kumar Mishra et al. 2011). Thus, Hub1 facilitates the alternative splicing of SRC1 gene by promoting efficient usage of non-canonical 5'ss (Shravan Kumar Mishra et al. 2011). Hub1 was also shown to bind and stimulate the ATPase activity of Prp5 (Karaduman et al. 2017). Sde2 is a new member of the family of UBL proteins. In humans and fission yeast, it is synthesized as an inactive precursor which gets processed by deubiquitinating enzymes (Jo et al. 2016; Thakran et al. 2018). In fission yeast, the processed Sde2 is required for the splicing of a subset of genes in an intron-specific manner (Thakran et al. 2018). Thus, UBLs act as specialized splicing regulators in the process of pre-mRNA splicing.

1.2.6 Heterochromatin

Eukaryotic genomic DNA is organized in the nucleus with the help of histones and non-histone proteins. This organization is called chromatin and each chromatin or nucleosome consists of 146 bp of DNA organized around histones (Luger et al. 1997). The compaction of chromatin varies on different region of the genome. Euchromatin is transcriptionally-active, rich in gene and less condensed. Heterochromatin is highly condensed and mostly consists of repetitive DNA elements like satellite sequences and transposons (Huisinga, Brower-Toland, and Elgin 2006). So, heterochromatin prevent the jumping of transposons which can lead to genome instability. It also regulates the transcription of genes in a cell-type specific manner and is required for proper centromeric and telomeric function (Allshire and Madhani 2018).

The fission yeast *Schizosaccharomyces pombe* has served as a useful model organism for studying the formation and maintenance of heterochromatin as it contains large heterochromatic regions despite having a small genome. And the heterochromatic pathways are conserved between fission yeast and humans (Grewal and Jia 2007; Martienssen, Zaratiegui, and Goto 2005). Heterochromatin regions are defined by distinct histone modifications. The tails of histones protrude from the nucleosomes and are enriched in lysine. These lysine residues are subjected to various post-translational modifications (PTM) by enzymes which can be termed as 'writers'. These modifications are bound by specialized proteins which contains the 'reader' domains. And these PTMs can be removed by 'eraser' proteins (Figure 1.2.9) (Allshire and Madhani 2018).

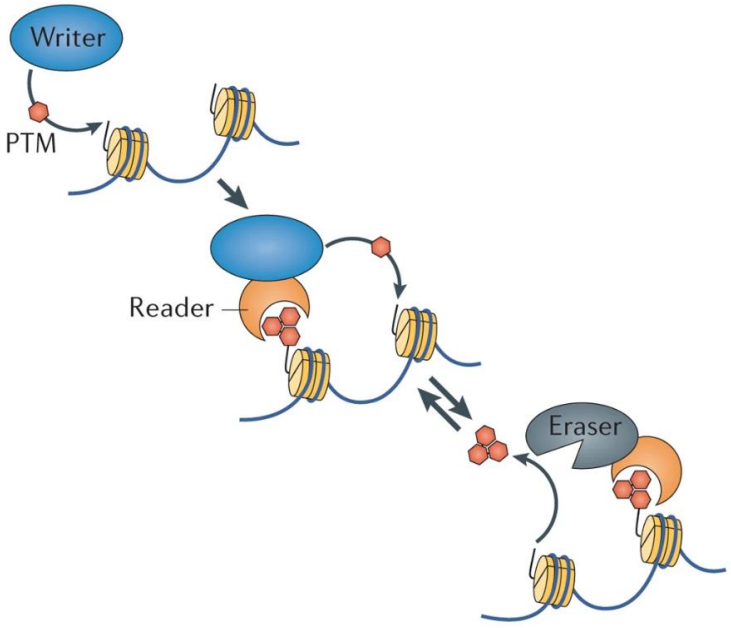


Figure 1.2.9: Mechanism of heterochromatin modifications.

The functioning of different 'readers', 'writers', and 'erasers' in catalyzing the PTMs on the histones. Taken from the reference (Allshire and Madhani 2018).

Heterochromatin regions are generally characterized by hypoacetylation and hypermethylation at histone H3 lysine 9 (Noma, Allis, and Grewal 2001). For the initiation of heterochromatin, the DNA-binding proteins that binds to specific sequences or non-coding RNAs from the nucleation centre recruit histone deacetylases (HDACs) and the H3K9 methyltransferase (HMT), Clr4 (Jia, Noma, and Grewal 2004; T. A. Volpe et al. 2002). This leads to the methylation of H3K9. Interestingly, Clr4 also contains a chromodomain that can bind to methylated histones. Clr4's ability to 'read' and 'write' histones forms a self-propagating mechanism that can methylate the adjacent nucleosomes also (K. Zhang et al. 2008). These methylated histones are then bound by chromodomain-containing protein, Swi6/HP-1, which then recruits more chromatin modifiers (Bannister et al. 2001)(Lachner et al. 2001)(Nakayama et al. 2001)(M David Stewart, Li, and Wong 2005). All these enzyme activities lead to the establishment and maintenance of heterochromatin in large domains independent of DNA sequences (Jiyong Wang, Jia, and Jia 2016).

Splicing factors have also been shown to play an important role in heterochromatin silencing through their splicing-independent activity. Splicing factors are involved in the generation of centromeric siRNAs and also associate with the RNAi machinery which points to their role in heterochromatin assembly independent of their splicing activity (Bayne et al. 2008). The U4 snRNA has also been shown to regulate the heterochromatin formation at the centromeres (Chinen et al. 2010). However, the splicing defects of key heterochromatin factors have also been observed in splicing factors mutants. This can explain the heterochromatin defects observed in the loss of several splicing factors

(Bayne et al. 2014; Kallgren et al. 2014; Jiyong Wang et al. 2014). So, splicing factors play an important role in regulating heterochromatin assembly through their splicing and non-splicing activity.

1.3 PRELUDE TO THE STUDY

Splicing of precursor mRNAs into protein-coding mRNAs is an essential step in gene expression. It also promotes regulated gene expression and alternative splicing. The process is completed by the spliceosome assembled from five small nuclear RNA-protein complexes (snRNP). Spliceosomes excise pre-mRNA introns detected by their splicing signals. The 5' splice site (5'ss) is detected by U1 snRNP, while the branchpoint (BP), the poly-pyrimidine tract, and the 3' splice site (3'ss) are detected by U2 snRNP and its accessory factors (U2AF) (Plaschka et al. 2018; Reich et al. 1992). Further, the U4/U6-U5 tri-snRNPs get recruited to form the B complex spliceosome (Wahl, Will, and Lührmann 2009; Will and Lührmann 2011). The unwinding of U4/U6 snRNAs by Brr2 helicase mediates the formation of the U2/U6 snRNP complex. The active site embedded within the U2/U6-U5 snRNP complex is formed with two catalytic metal binding sites in U6 snRNA, which catalyzes both transesterification reactions needed to excise the intron and ligate the two exons (Bertram et al. 2017; Fica et al. 2017, 2019a; Yan et al. 2016).

Introns differ in positions and sequences of splicing signals, lengths, and location in the pre-mRNA. Furthermore, the presence of sequence motifs that act as splicing enhancers or silencers, and the propensity to form secondary structures, also differ (Hooks, Delneri, and Griffiths-Jones 2014; Kupfer et al. 2004; S.K. Mishra and Thakran 2018; Taggart et al. 2017; William Roy and Gilbert 2006). This diversity is critical for regulated gene expression and alternative splicing; therefore, intron-rich eukaryotes experience an enormous impact of RNA splicing on their physiology. To tackle the processing of diverse introns, intron-specific splicing regulators that include RNP assembly factors, RNA binding proteins, or RNA and protein-modifying proteins and enzymes are recruited to the spliceosome (Y. Lee and Rio 2015).

Ubiquitin and ubiquitin-like proteins (for example, SUMO, Hub1, and Sde2; collectively referred to as UBLs) are post-translational modifiers of proteins controlling diverse cellular activities. They also regulate pre-mRNA splicing by modifying spliceosomes, thereby potentiating the machinery to act on specific introns and pre-mRNAs (Chanarat and Mishra 2018). UBLs appear dispensable for constitutive pre-mRNA splicing involving introns with canonical splicing signals but become critical for excising introns with noncanonical sites. For example, the UBL Hub1/UBL5 is conserved from *S. cerevisiae* to humans. It promotes alternative splicing and facilitates spliceosomal recognition of weaker 5'ss. It modifies spliceosomes by binding to HIND-containing splicing factors (Ammon et al. 2014; Karaduman et al. 2017; Shravan Kumar Mishra et al. 2011; Wilkinson et al. 2004). *S. cerevisiae* Hub1 also activates the spliceosomal RNA helicase Prp5, allowing Hub1-modified spliceosomes to use noncanonical 5'ss and allow alternative splicing (Karaduman et al. 2017).

Spliceosomes modified by the ubiquitin-fold-activated Sde2 promote the excision of selected introns in a subset of pre-mRNAs in *S. pombe*. This splicing regulator is conserved among intron-rich

eukaryotes up to humans but is absent in intron-poor organisms such as *S. cerevisiae*. Sde2 was recently shown to be important in pre-mRNA splicing in mammalian cells (Floro et al. 2021). Sde2 is translated as an inactive precursor harbouring an N-terminal UBL ($Sde2_{UBL}$) that gets processed by deubiquitinating enzymes (DUBs) in a conserved GG~K motif to form activated ^{K}Sde2-C (Jo et al. 2016; Thakran et al. 2018). Following processing by DUBs, Ubp5 and Ubp15 in *S. pombe*, Sde2 matures into spliceosomal competent ^{K}Sde2-C with lysine at the N-terminus. The removal of $Sde2_{UBL}$ and the free lysine of ^{K}Sde2-C are critical for its intron-specific splicing function. However, the question 'why only selected pre-mRNAs required Sde2 for efficient splicing' remained unanswered.

1.4 RESULTS

1.4.1 Sde2 is a ubiquitin-fold activated splicing regulator

In our lab, it was previously reported that *Schizosaccharomyces pombe* Sde2 is a ubiquitin-fold-activated splicing regulator that facilitates pre-mRNA splicing in an intron-specific manner (Thakran et al. 2018) (Figure 1.4.1A). Splicing-sensitive microarrays were performed with wildtype (wt) and Δ*sde2* strain to understand the role of Sde2 in pre-mRNA splicing. The microarray data, further verified by RT-PCR, showed that the lack of Sde2 (Δ*sde2*) leads to the retention of only selected introns in a subset of multi-intronic genes (Thakran et al. 2018). A key splicing target of Sde2 is *rap1* pre-mRNA encoding the telomere-binding shelterin complex subunit (Fujita, Tanaka, and Kanoh 2012). We performed RT-PCR experiments and western blot analysis in wildtype and *Δsde2*. For RT-PCR experiments, we isolated total RNA from wildtype and *Δsde2* strains. We performed cDNA synthesis and did RT-PCR assays with primers binding on the exon-1 and exon-3 of *rap1* gene (Figure 1.4.1B). We observed a specific retention of intron-2 of *rap1* (*rap1-i2*) in the absence of Sde2. We also performed western blot analysis to check for the protein levels of Rap1. We tagged Rap1 chromosomally using a C-terminal 6HA tag in wildtype and *Δsde2* strains and did western blot for Rap1 using anti-HA antibody (Figure 1.4.1C). The Rap1 levels were lower in *Δsde2* compared to wt. Thus, Rap1 is a key splicing target of Sde2.

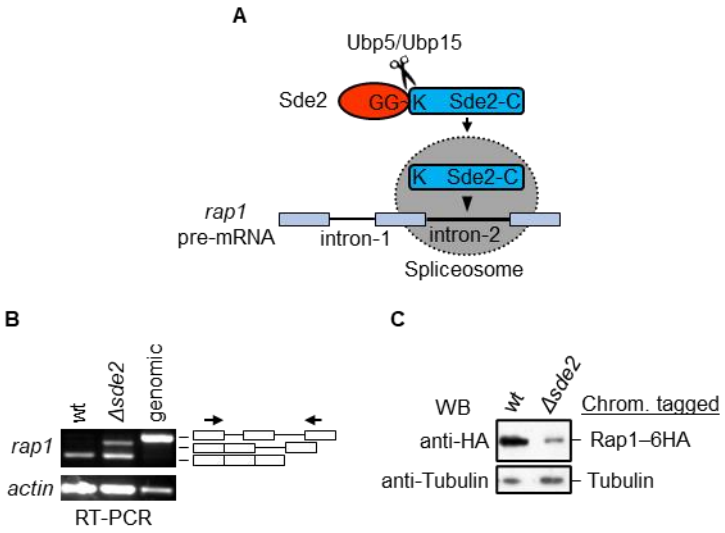


Figure 1.4.1: Splicing of *rap1-i2* is dependent on Sde2.

A) Schematic of Sde2 activation and function for the splicing of *rap1-i2*. B) Semi-quantitative RT-PCR shows retention of *rap1-i2* in *Δsde2* strain. Arrows, blocks and lines indicate the primers, exons and introns, respectively. *S. pombe* genomic DNA was used as a control to size intron containing transcript. C) Rap1 protein level is lower in *Δsde2* strain compared to wt. Immunoblot analysis was performed to detect chromosomally 6HA epitope-tagged Rap1 using anti-HA antibody.

1.4.2 Split-*ura4* reporter

The question then arises why only specific introns, such as *rap1's* intron-2 but not its intron-1, require Sde2 for splicing. To understand the intron specificity of Sde2, we made two splicing reporters with the *S. pombe ura4* gene split individually by the two *rap1* introns (Figure 1.4.2A). We tested the reporters' ability to complement a *ura4* auxotrophic *S. pombe* strain. Functional *ura4* mRNA and protein were expected only after accurate splicing of the pre-mRNAs that would allow the cells to grow on plates lacking uracil (−URA), but not on uracil-counter selection plates containing 5-fluoroorotic acid (+FOA) (Figure 1.4.2B). With the reporter split by the Sde2-independent *rap1* intron-1 (*rap1-i1* reporter), both the wt and Δ*sde2* strains grew on −URA plate, but not on +FOA. On the contrary, with the reporter split by the Sde2-dependent *rap1* intron-2 (*rap1-i2* reporter), wt cells grew on −URA, but not on the counter selection plate containing FOA, while Δ*sde2* cells did not grow well on −URA, but grew on the +FOA plate (Figure 1.4.2C).

The above results suggest that *rap1* intron-2 (but not intron-1) requires Sde2 for splicing. These assays also ruled out the possible involvement of transcription, UTRs or the protein-coding parts of the *rap1* gene for its Sde2 dependency. Thus, the splicing role of Sde2 was attributed to certain features in the intron.

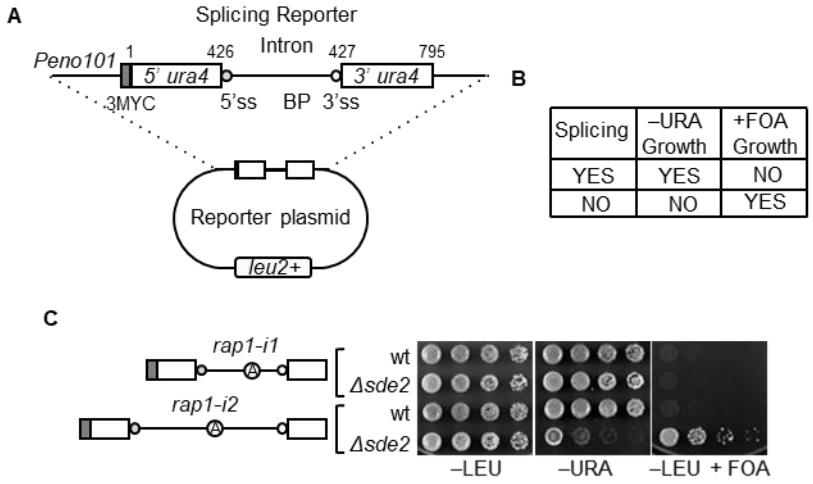


Figure 1.4.2: Split-*ura4* reporter.

A) Design of splicing reporters (ss, splice site; BP, branch point). Numerical on the reporter shows insertion site of introns in *ura4* gene. B) Table showing the expected result from the reporter in *S. pombe*. C) Splicing-proficient strain grows on –URA plate but does not grow on +FOA (5-fluoroorotic acid) plates, and vice-versa (Data obtained from Karan).

1.4.3 Ubiquitin-like processing of Sde2 is essential for its splicing activity

Sde2 precursors in *S. pombe* and humans are processed into $Sde2_{UBL}$ and ^{K}Sde2-C (Jo et al. 2016; Thakran et al. 2018). After the deubiquitinating enzymes (DUBs), Ubp5 and Ubp15, cleave the *S. pombe* precursor at the conserved GG~K motif, the processed ^{K}Sde2-C enters the spliceosome and performs its function. ^{K}Sde2-C is short-lived due to its proteasomal degradation by the N-end rule

pathway (Rageul et al. 2019; Thakran et al. 2018). We tested the importance of ubiquitin-like processing for reporter splicing in three strains: (i) DUB deletion strain, Δ*ubp5* Δ*ubp15,* (ii) a processing defective *sde2 (AAK)* mutant (amino acids changes underlined), and (iii) a ubiquitin–^{K}Sde2-C chimera that replaces chromosomal *sde2.* Similar to the Δ*sde2* strain, the *sde2* processing defective strains Δ*ubp5* Δ*ubp15* and *sde2 (AAK)* spliced only the *rap1-i1* reporter and became uracil positive. But both the strains could not splice the *rap1-i2* reporter and remained uracil negative**.** On the contrary, the Sde2-replacing ubiquitin–Sde2-C chimeric strain spliced both reporters and became uracil positive (Figure 1.4.3A).

We also tested the need for N-terminal lysine in ^{K}Sde2-C in a lysine-to-methionine mutant strain *sde2 (GGM)*. This ^{M}Sde2-C protein is stable as it is not a N-end rule substrate. This mutant, which forms ^{M}Sde2-C, could splice only the *rap1-i1* reporter but not *rap1-i2* (Figure 1.4.3B). Thus, both ubiquitin-like processing and N-terminal lysine of ^{K}Sde2-C are essential for the intron-specific splicing activity of Sde2. We also checked if the different chimeras complemented the growth defects in the Δ*sde2* strain. Only the ubiquitin–^{K}Sde2-C chimera complemented growth defects in the Δ*sde2* strain, suggesting ubiquitin could replace the $Sde2_{UBL}$ activity of producing functional ^{K}Sde2-C (Figure 1.4.3C).

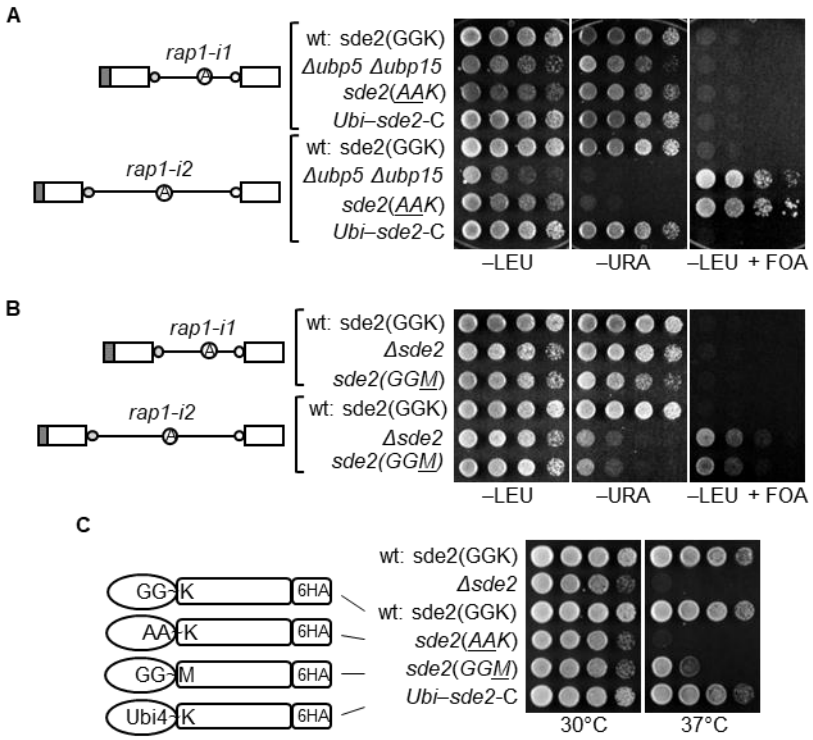


Figure 1.4.3: Ubiquitin-like processing of Sde2 leading to the generation of ^{K}Sde2-C is important for its splicing activity.

A) Ubiquitin-like processing of Sde2 is essential for its intron-specific splicing activity. The processing defective strains, *Δubp5 Δubp15* and *sde2(AAK)*, could not splice *rap1-i2* reporter, whereas the ubiquitin–^{K}Sde2-C chimeric strain *(Ubi–sde2-C)* spliced the reporter. B) The N-terminal lysine of ^{K}Sde2-C is crucial for intron-specific splicing activity of Sde2. sde2(GGM) mutant strain was not able to splice *rap1-i2*. C) Growth assay of Sde2 variants at different temperatures.

1.4.4 Sde2-dependent introns have longer distance between Branch point (BP) and 3' splice site (3'ss)

Next, we ask which features of the intron targets detected in the microarrays made them dependent on Sde2? Data obtained from splicing-sensitive microarray performed previously in our lab was analyzed (Thakran et al. 2018). The target introns (defined as $\log_2$ Δ*sde2*/wt signal ratio at 30ºC ≥0.5) lacked a common sequence motif and did not match with respect to the strengths of their splicing signals, lengths, or position in resident pre-mRNAs. After further scrutiny of the Sde2-target introns, we noticed that the spacing between BP and 3'ss in most of them was longer than the average spacing of introns in *S. pombe* (Figure 1.4.4). The spacing is defined as the RNA bases between branch adenosine and 3'ss. BP and 3'ss in *S. pombe* introns are on average 12 nt apart from each other (Kupfer et al. 2004). For example, BP and 3'ss are 39 nt apart in *rap1* intron-2 but only 14 nt apart in *rap1* intron-1.

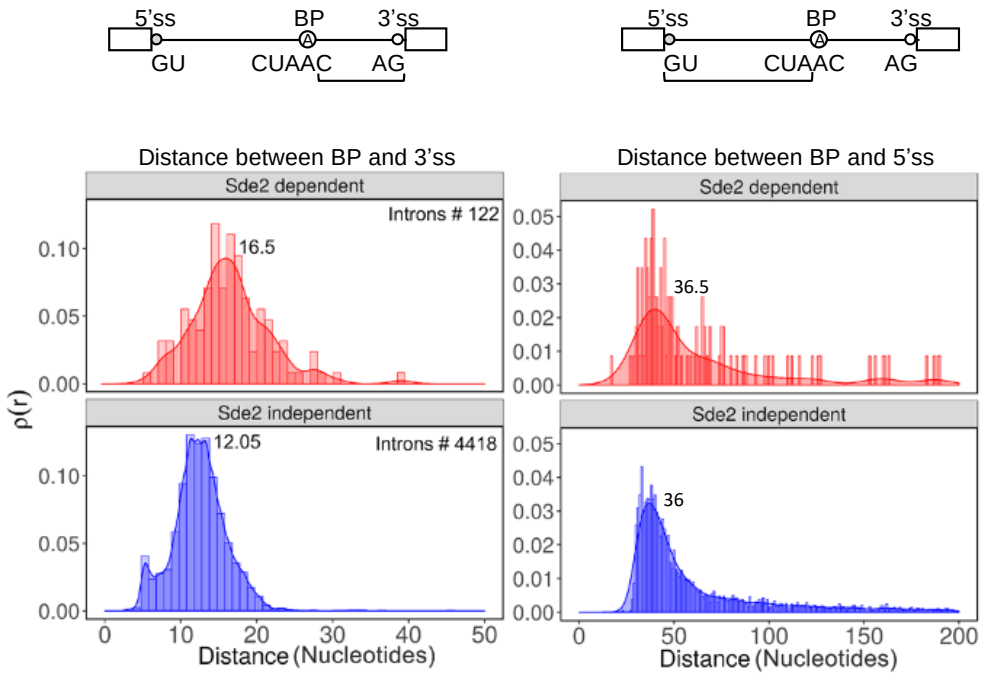


Figure 1.4.4: Bioinformatic analysis of Sde2-dependent introns.

Distribution of distances (number of nucleotides) between the branch point (BP) adenosine and the 5' and 3'-splice sites (ss) of the Sde2-dependent and -independent introns (Sde2-dependent ≥ 0.5 Log2 Δ*sde2* / wt ratio at 30ºC; Sde2-independent ≤ Log2 Δ*sde2* / wt ratio at 30ºC). Red peaks and histograms show 122 Sde2-dependent introns, and blue peaks and histograms show 4418 Sde2-independent introns. The numbers on the peaks show their maxima. (Microarray data analysis performed by Dr. Monika Sharma).

1.4.5 Reduction of distance between BP-3'ss makes introns independent of Sde2

To test the importance of a longer BP-3'ss in Sde2-dependent introns, we made a *ura4* reporter with intron-3 of *ftp105.* Ftp105 is a Golgi localized protein (Kouranti et al. 2010). The splicing of *ftp105-i3* is dependent on Sde2 (Figure 1.4.5A). The distance between BP and 3'ss of *ftp105-i3* is 24 nt. We reduced the BP-3'ss spacing in the *ftp105-i3* reporter by bringing its BP closer at 12 nt to 3'ss. Interestingly, this intron got excised in Δ*sde2* strain, as monitored by the growth on −URA plate and the lack of growth on +FOA plate (Figure 1.4.5B). We also performed western blot as the *ura4* reporter has N-terminal 3MYC tag. The defect in the protein level of Ura4 was also rescued when the BP-3'ss spacing was reduced (Figure 1.4.5D). We also made a mutant *ura4* reporter with a deletion of a nucleotide at 786th position which led to frame shift and addition of 40aa to the full length *ura4* making it a hypomorphic mutant. We used this mutated reporter to make the assay more sensitive. We monitored the growth in −URA plate and also performed western blot analysis (Figure 1.4.5C, E). We observed a similar rescue as in Figure 1.4.5B.

We made reporters with more target introns like, *rap1* intron-2 (Figure 1.4.5F) and *pyp3* intron-1 (Figure 1.4.5G) and observed a similar Sde2 dependency for both. To verify if the longer spacings

between BP and 3’ss made them also Sde2-dependent, we reduced the gap in *rap1 intron-2* from 39 nt to 12 nt. Sde2 was not needed for the excision of this intron variant. Thus, Sde2-dependent introns have a longer spacing between BP-3’ss which on reducing make them Sde2 independent.

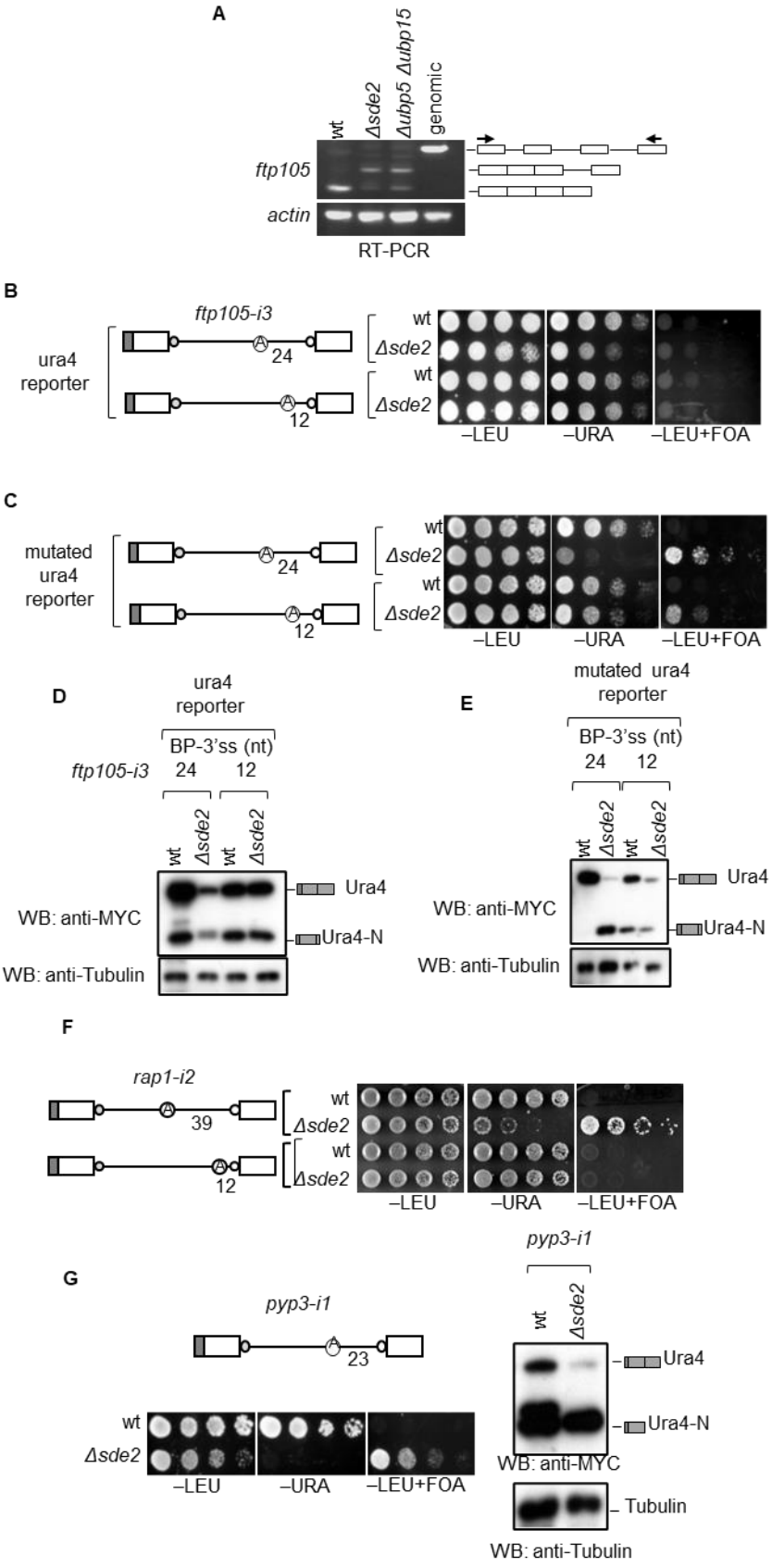


Figure 1.4.5: Sde2 target introns have longer spacing between the BP and 3′ss.

A) Semi-quantitative RT-PCR shows retention of *ftp105-i3* in indicated strain. Arrows, blocks and lines indicate the primers, exons and introns, respectively. *S. pombe* genomic DNA was used as a control to size intron containing transcript. B) and D) Growth and immunoblot of the indicated strains with the *ftp105-i3* reporter with different distance between BP and 3'ss. C) and E) Growth and immunoblot of the indicated strains with the mutated *ftp105-i3* reporter with different distance between BP and 3'ss. F) Growth assay showing *rap1-i2* with 39 nt between BP and 3'ss was Sde2-dependent. The reduction of this spacing made its splicing Sde2-independent (Data obtained by Karan). G) Growth and immunoblot of the indicated strains with the *pyp3-i1* reporter (Incubation time: -LEU, -URA - 4 days; +FOA - 4 days).

1.4.6 Selection of BP-distant 3'ss is not a dominant phenomenon

We next tested whether Sde2 could enhance splicing through BP-distant 3'ss in the presence of a BP-near 3'ss. For this, we made a reporter with a Sde2-independent intron, *tho5-i1*. The distance between BP and 3'ss of *tho5-i1* is 12 nt. In this reporter, we introduced two competing 3'ss, BP-near (12 nt) and BP-distant (21 nt), and monitored its splicing in wt cells. We performed growth assays, RT-PCR assays and western blot analysis for these constructs (Figure 1.4.6). A protein corresponding to the mRNA formed after the usage of only the BP-near 3'ss could be detected, suggesting that BP-distant splicing is avoided in the presence of a more favorable BP-near 3'ss.

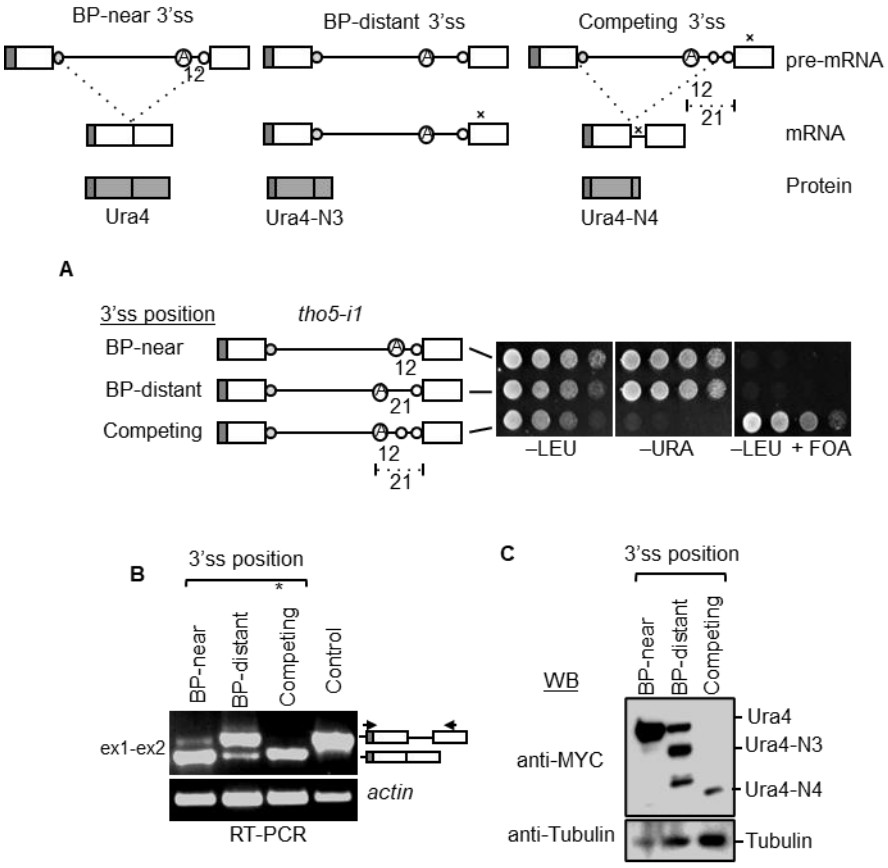


Figure 1.4.6: Spliceosome prefers BP-near 3'ss over BP-distant ones.

A) Splicing of *tho5-i1* reporters with competing 3'ss (BP-near and BP-distant) in wt was tested on –URA and +FOA plates (incubation time: -LEU, -URA - 3 days; +FOA - 4 days). B) Semiquantitative RT-PCR monitoring splicing of BP-near and BP-distant 3'ss in the *tho5-i1* reporter. Primers are as shown in the block diagram (ex-exon). * marks the band that appears due to the use of BP-near 3'ss, which leads to the incorporation of nine additional nucleotides in the ura4 cDNA transcript. C) Western blot analysis shows the translational product of mRNA spliced using BP-near 3'ss but not from BP-distant 3'ss. Ura4 -N3 and -N4 indicate proteins from mRNAs with exon-1 until the stop codon. Asterisk (*) mark proteins arising possibly from aberrant splicing or proteolytic cleavage.

1.4.7 Sde2, Cay1 and Tls1 control the expression of RNA interference (RNAi) and heterochromatin factors via splicing

In our lab, Karan performed a screen to identify additional splicing factors similar to Sde2. Splicing of *rap1-i1* and *rap1-i2* reporters were tested in a collection of forty-eight viable deletion mutants of putative splicing factors from Bioneer deletion library. From the screen, we identified Cay1 and Tls1 as additional intron-specific splicing factors as they are required for the splicing of only *rap1-i2*.

The above mentioned proteins have also been involved in heterochromatin silencing (Sugioka-Sugiyama and Sugiyama 2011)(Lorenzi et al. 2015; Jiyong Wang et al. 2014). In contrast, splicing factors have also been shown to act in heterochromatin silencing independently of their splicing

functions (Bayne et al. 2008; Bernard et al. 2010; Chinen et al. 2010). We hypothesized that these factors could regulate heterochromatin formation via splicing. Thus, we monitored protein levels of selected Sde2 targets functioning at the chromatin level. A 6HA epitope tag was inserted at the C-termini of the genes at their chromosomal loci in *S. pombe*. The genes *rap1*, *hif2*

, and *dsh1* were tagged in wt, Δ*sde2*, Δ*cay1*, and Δ*tls1* strains. Anti-HA western blots showed diminished protein levels of key heterochromatin factors (Rap1, RNAi factor Dsh1 (Kawakami et al. 2012); Set3 histone deacetylase complex protein Hif2 (Rentas et al. 2012) in all three mutants (Figure 1.4.7A). RT-PCR assays showed retention of specific introns in the above heterochromatin and RNAi factors in Δ*sde2*, Δ*cay1*, and Δ*tls1* strains (Figure 1.4.7B). Thus, intron-specific splicing factors regulate heterochromatin assembly through its splicing activity.

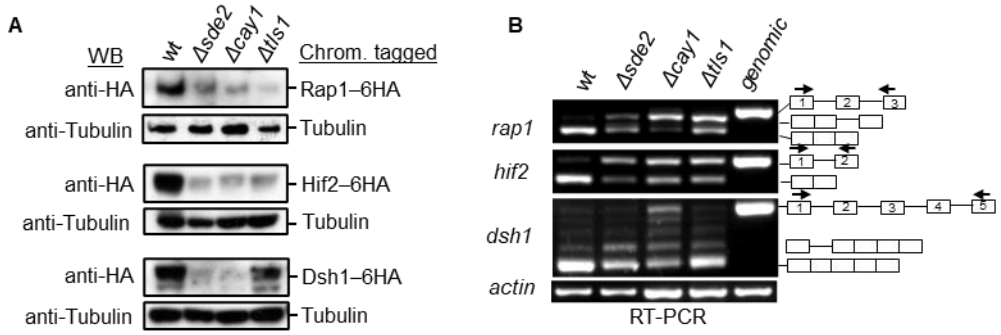


Figure 1.4.7: Sde2, Cay1, and Tls1 control the expression of heterochromatin factors.

A) Rap1, Hif2, and Dsh1 proteins are lower in deletion mutants of intron-specific splicing factors. Immunoblotting was performed for the chromosomally C-terminal 6HA-tagged strains using an anti-HA antibody. B) Semiquantitative RT-PCR was performed to assay the splicing of heterochromatin factors in the indicated strains. Arrows in the schematic indicate where the primer binds (Data obtained from Anupa).

1.4.8 Alternative splicing mediated by intron-specific splicing factors

We then checked the protein levels of additional targets of Sde2 like Mcs2 (Molz et al. 1989), Hif2, Psf3 (Carrozza et al. 2005), Rxt2, and Rap1 (Fujita, Tanaka, and Kanoh 2012) which were monitored by expressing N-terminal 3MYC epitope-tagged genomic constructs in wt and Δ*sde2* strains. These proteins were lower in the Δ*sde2* strain, further confirming the role of Sde2 in gene expression. Expectedly, the full-length Rap1 protein diminished in Δ*sde2*, but an alternate Rap1 protein of ~19 kDa (Rap1-N) was detected in this strain. Similarly, the full-length Rxt2 protein decreased in Δ*sde2*, but an alternate form of ~13 kDa accumulated (Figure 1.4.8A). These results indicated that controlled BP-distant 3'ss usage could promote alternative splicing through intron retention.

We then checked if Rap1-N is formed in the absence of Cay1 and Tlsl. Indeed, Rap1-N is formed in the absence of all intron-specific factors (Figure 1.4.8B). Rap1-N is expected to be translated from the intron-2-retained mRNA variant with a translational stop codon in the intron. We confirmed

Rap1-N's origin by mutating the stop codon. The change abolished the protein (Figure 1.4.8C). Therefore, the alternative protein would have the N-terminal 157 amino acids of Rap1 containing the BRCT domain and a part of the Myb domain (Fujita, Tanaka, and Kanoh 2012). A function to this segment of Rap1 protein has not been assigned.

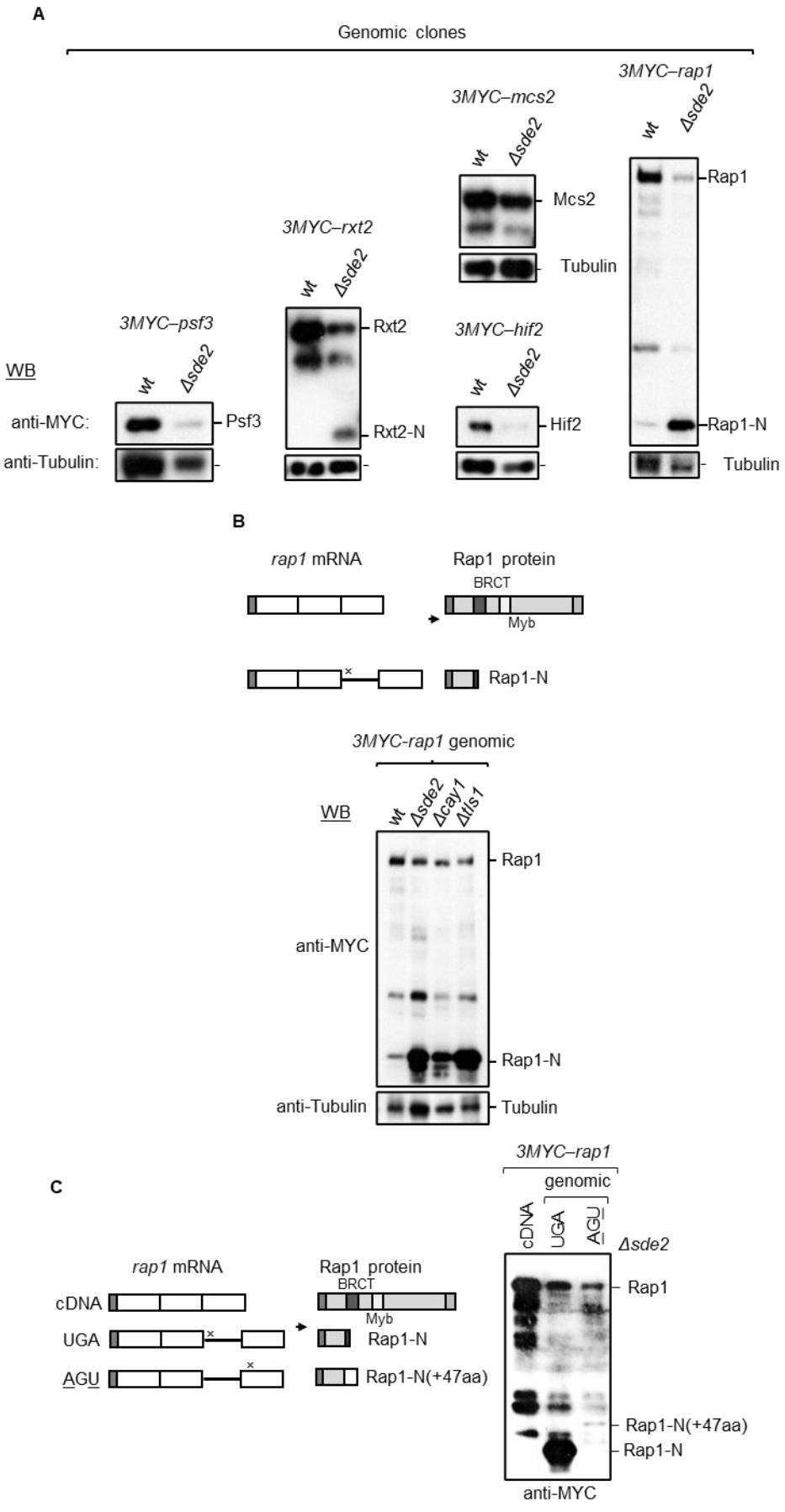

A
Genomic clones
3MYC–mcs2
3MYC–rap1
wt
Δsde2
Mcs2
Tubulin
Rap1
3MYC–rxt2
3MYC–psf3
3MYC–hif2
WB
anti-MYC:
anti-Tubulin:
Psf3
Rxt2
Rxt2-N
Hif2
Rap1-N
B
rap1 mRNA
Rap1 protein
BRCT
Myb
Rap1-N
3MYC-rap1 genomic
Δcay1
Δtls1
anti-MYC
anti-Tubulin
C
cDNA
UGA
AGU
Rap1-N(+47aa)

Figure 1.4.8: Alternative splicing of proteins through intron retention.

A) Protein analysis show levels of Mcs2, Hif2, Psf3, Rxt2 and Rap1 in *Δsde2* deletion strain compared to the wt strain. Both Rap1 and Rxt2 proteins showed alternative forms in *Δsde2* strain. Genomic constructs were expressed under *nmt81* promoter B) An alternative form of Rap1 protein (Rap1-N) accumulated in the deletion mutants. A genomic construct of Rap1 under *eno101* promoter was expressed in the mutants. C) Rap1-N is formed because of intron-2 retention. × marks the stop codon arising during the translation of the intron-retained mRNA. Western blot with the stop codon mutated intron-2 version of *rap1* genomic construct. Instead of Rap1-N, an upshifted band was observed because of a new stop codon in exon-2.

Thus, intronic diversity and dedicated splicing regulators of the kind discussed in this study allow the cell to control gene expression and promote alternative splicing to create protein variants that help the cell under specific conditions.

1.5 DISCUSSION

1.5.1 The regulators and mechanism of BP-distant 3′ss usage in *S. pombe*

In this chapter, we found that Sde2 facilitate the use of BP-distant 3′ss. The splicing targets of Sde2 were known, but the reason for its intron-specificity was unknown. We monitored the activities of *S. pombe ura4* splicing reporters harboring introns with a BP-near and BP-distant 3′ss to study its intron-specific splicing function. A similar reporter with *nda3* introns was used to study exon skipping in *S. pombe* (Haraguchi et al. 2007). The reporters were assayed by three techniques: growth, RT–PCR and western blot, which collectively made the outcome more sensitive and specific. We observed that the ubiquitin-like processing of precursor Sde2 and N-terminal lysine of ^{K}Sde2-C is essential for the intron-specific splicing activity of Sde2. The fusion of ubiquitin-fold and its processing facilitates the generation of Sde2-C starting with a lysine. In our lab, we have shown that activated ^{K}Sde2-C facilitates association of spliceosomes with another intron-specific splicing factor Cactin/Cay1. Although the lysine does not appear critical for the interaction of ^{K}Sde2-C with spliceosomes, the residue is important for interactions with Cactin (Thakran et al. 2018). The free lysine in ^{K}Sde2-C was also shown to play a regulatory role, as it makes the protein a substrate of the N-end rule pathway of proteasomal degradation. Thus, the removal of Sde2-UBL and the free lysine of ^{K}Sde2-C are critical for its intron-specific splicing function. The splicing with a BP-distant 3′ss exons became independent of Sde2 once the spacing between the BP and 3′ss was reduced. We also characterized additional intron-specific splicing factors, Cay1 and Tls1.

The RNA between the BP and 3′ss must loop during exon ligation to juxtapose the two exons in the catalytic centre. This RNA structure in the spliceosome is yet to be seen (Fica et al. 2017, 2019b; Yan et al. 2016), but its flexible or longer trajectories could hinder the spliceosome by slowing down the docking of the incoming 3′ss in the catalytic centre. We propose that these factors could guide the incoming 3′ss towards the spliceosome's catalytic centre. Sde2 promotes the recruitment of Cay1 in the spliceosome (Fica et al. 2019b; Thakran et al. 2018). This result was supported by the cryo-electron microscopy structure of the human post-catalytic spliceosome. The structure shows that Sde2-C and Cactin function as exon ligation factors by stabilizing the branch helix in a suitable conformation for the ligation (Fica et al. 2019b). Tls1 has been reported to bind Brr2 (a U5-snRNP-specific RNA helicase that unwinds U4/U6 snRNA duplexes during spliceosomal activation) in *S. pombe* and humans (Bergfort et al. 2022; Jiyong Wang et al. 2014). Tls1 has been shown to regulate Brr2's activity in humans (Bergfort et al. 2022). A Brr2 mutant showed second step splicing defects for BP-distant 3′ss introns and introns with potential secondary structures between the BP and 3′ss. The Brr2 helicase has been proposed to play an additional role in the second step of splicing via substrate repositioning (Hahn et al. 2012). Thus, Sde2, Cay1 and Tls1 may facilitate the positioning of the RNA intervening the BP and 3′ss (Figure 1.5.1).

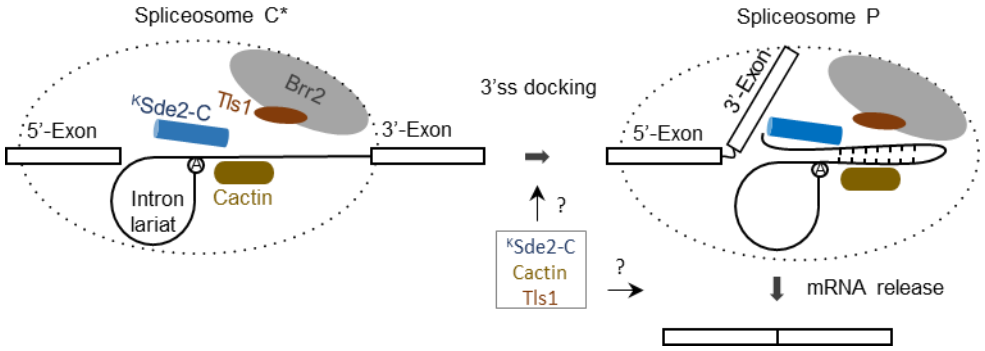


Figure 1.5.1: Proposed mechanism of intron-specific splicing factors Sde2, Cactin, and Tls1.

Sde2 is shown to be present in the C* complex in the cryo-EM structure of the human spliceosome. Sde2 recruits Cactin into the spliceosome. Tls1 interacts with Brr2, a U5 snRNP specific RNA helicase, in humans and *S. pombe*. Here, we propose that Sde2 recruits Cactin, stabilizing the RNA between BP and 3'ss and therefore bridging the gap between BP and 3'ss. Tls1, on the other hand, might regulate Brr2's activity which could be important for splicing the BP-distant 3'ss introns. These factors can also play a role as mRNA release factors.

1.5.2 Role of intron-specific splicing factors in the selection of 3'ss

Mechanism of selection of 3'ss remains poorly understood. Although there are many models which explain how the selection of 3'ss is made such as scanning mechanism model but how the exact mechanism of 3'ss selection still remains unclear (Smith et al. 1989). In our study, we found that the intrinsic choice of the spliceosome for a BP-near 3′ss made by essential RNA-binding proteins (Agrawal et al. 2016; Banerjee et al. 2013; Chua and Reed 2001; Shao et al. 2014; Vijaykrishna et al. 2016; S. Wu et al. 1999) dominated over the activities of these regulators; a BP-near 3′ss was preferred even in the presence of Sde2, Cay1 and Tls1. However, study with minigene reporters in humans have shown that the distance between two 3'ss in an intron can affect the choice of 3'ss which is used (Chua and Reed 2001). Therefore, our assays do not completely rule out the regulatory activity of these factors on competing 3'ss selection. In humans, C9ORF78, the homolog of Tls1, regulates the splice site usage in NAGNAG introns(NAGNAG introns has two tandem repeats of 3'ss (Bergfort et al. 2022)). Taken together, these intron-specific splicing factors might help in the regulating the selection of 3'ss.

1.5.3 The physiological relevance of splicing through BP-distant 3′ss

We report that Sde2, Cay1 and Tls1 control gene expression and alternative splicing of selected chromatin and RNAi factors in *S. pombe*. We and others have shown that their mutants are defective in heterochromatin silencing and genomic instability (Anandarajan et al. 2020; Bernard et al. 2010; Thakran et al. 2018; Jiyong Wang et al. 2014). RNAi defects in the *sde2* mutant have also been reported (Bernard et al. 2010). These phenotypes could result from defects in the expression of chromatin factors, e.g. the shelterin complex subunit Rap1, the histone deacetylases Hif2 and Rxt2, or the RNAi machinery

assembly factor, Dsh1. The Sde2 mutant also accumulated alternative forms of Rap1 and Rxt2. The function of the alternative Rap1 and Rxt2 has not yet been elucidated. Although their aberrant origin cannot be ruled out, these proteins might function only under specific conditions. Also, the intron-containing transcripts which accumulates in the absence of these splicing factors might also function in heterochromatin silencing. RNAs have been shown to play an important role in regulating the heterochromatin assembly (Loda and Heard 2019; T. Volpe and Martienssen 2011). So, it will be interesting to check if these RNA transcripts have any role in regulating heterochromatin assembly.

Sde2, Cay1 and Tls1 could also make gene expression and alternative splicing conditional or tissue specific in multicellular eukaryotes rich in diverse introns. Indeed, mammalian introns with BP-distant 3′ss have a higher tendency to undergo alternative splicing (Akerman and Mandel-Gutfreund 2007; Gooding et al. 2006). Further regulation of splicing by these factors is plausible, considering a stringent control over Sde2 protein, involving its activation by DUB and degradation by the proteasome (Jo et al. 2016; Rageul et al. 2019; Thakran et al. 2018).

1.6 CONCLUSION

In this chapter of my thesis, I found that Sde2 facilitates the excision of introns with longer spacing between the BP and 3’ss (referred to as BP-distant 3’ss). This activity requires the cleavage of $Sde2_{UBL}$ by DUB and the free lysine of processed ^{K}Sde2-C. Using splicing reporters made with the *ura4* gene split by introns of varying distances between the BP and 3’ss, we searched for *S. pombe* deletion mutants of putative splicing factors. We identified Cactin/Cay1 and Tls1 as additional regulators of BP-distant splicing. These three splicing regulators are absent in budding yeast but are conserved from the fission yeast *S. pombe* to metazoans. They regulate gene expression and alternative splicing of various heterochromatin factors, including the telomeric factor Rap1.

2 CHAPTER 2: Role of a Golgi-localized deubiquitinating enzyme module

2.1 ABSTRACT

In the previous chapter, I found that the spliceosomal competent ^{K}Sde2-C generated by the processing activities of the DUBs, Ubp5 and Ubp15, is required for the splicing of *ftp105* intron-3. Out of the 20 DUBs in *S.pombe*, Ubp5 is the only known DUB to localize to Golgi. The Golgi localization of Ubp5 requires Ftp105. The significance of the localization of Ubp5 at the Golgi is not understood. Golgi apparatus is the sorting station of the cell that sorts various proteins into the extracellular environments or into the endomembrane organelles. Accurate sorting of proteins is important for the proper functioning of the cells. Ubiquitination has been shown to act as an important signal for protein quality control at the Golgi. Although several E3 ubiquitin ligases have been identified and characterized at the Golgi, the role of the deubiquitinating enzyme (DUB) is not well explored. So, we were interested in understanding the significance of the Golgi-localization of Ubp5.

In the second part, we found that Ftp105 and Ubp5 interact with each other and localize at the Golgi. Ubp5 is epistatic over Ftp105. Using mass spectrometric analysis, we identified Ght5, a high-affinity glucose transporter as one of the substrates of Ftp105 and Ubp5. In glucose-limiting conditions, the loss of this DUB module led to the mislocalization of Ght5 and its subsequent degradation in the vacuole. We identified Pub1 as one of the E3 ligases and Rod1 and Aly3 as the arrestin-related trafficking adaptors, that facilitate the ubiquitination of Ght5. Thus, we identified the role of the Golgi-localized DUB module in regulating the localization of a glucose transporter by counteracting the activity of Pub1 and arrestin-related trafficking adaptors, Rod1 and Aly3.

2.2 INTRODUCTION

2.2.1 Ubiquitin

Post-translational modification of proteins is an important regulation that can modify the activity, stability, localization or the function of the proteins. Proteins can be modified either with chemical constituents such as methylation, phosphorylation, lipidation and glycosylation or with another polypeptide.

One of the major modifiers of the proteins is the ubiquitin. Ubiquitin is a highly conserved 76 amnio acid protein that acts as molecular tags on different proteins. It has an extremely compact structure which includes a β-grasp fold and a C-terminal extension of six amino acids ending with a diglycine motif (Vijay-Kumar, Bugg, and Cook 1987). The C-terminal glycine of ubiquitin is conjugated to the ε-amino group of a lysine residue in a target protein forming a stable isopeptide bond. Recently, unconventional ubiquitination has also been observed involving ester linkages of proteins, lipids and sugars (Dikic and Schulman 2022). In the canonical ubiquitination pathways, a cascade of three enzymes brings about the process in an ATP-dependent manner. The E1 activating enzyme forms a thiol ester linkage with the carboxyl group of G76, then the E2 conjugating enzyme carries the activated ubiquitin in the form of thiol ester and the E3 ligase transfers the ubiquitin from the E2 to the substrate. The ubiquitination can occur on a single lysine on a protein (monoubiquitination) or can occur on multiple lysines (poly-monoubiquitination). The seven lysine residues of ubiquitin along with its N terminus methionine can serve as attachment sites for the conjugation machinery to attach multiple chains. The linkages are classified as Lys48, Lys63, Lys11, Lys33, Lys6, Lys27, Lys29 and Met1 mono or polyubiquitylation chains (Peng et al. 2003)(Tokunaga et al. 2009). The chains of ubiquitin can be built using the same lysine or the methionine linkage which are termed as homotypic conjugates. In the heterotypic conjugates, ubiquitin chains in different residues are observed increasing the complexity of the signal (Ikeda and Dikic 2008). These different ubiquitin chains contribute to the *ubiquitin code* where the E1-E2-E3 machinery act as the writers (Komander and Rape 2012). The ubiquitin code is read by proteins containing the ubiquitin binding domains (UBD). Depending on the ubiquitin chain, different UBD containing proteins interact with the ubiquitin chain leading to a specific biological outcome (Dikic, Wakatsuki, and Walters 2009). The ubiquitin code can be erased by a group of proteins called the deubiquitinating enymes (DUBs) (Figure 2.2.1). Like the UBD containing proteins, DUBs also exhibit specificity towards toward chain linkages (Komander, Clague, and Urbé 2009).

All the E3 ligases, UBD containing proteins and DUBs work together to produce a concerted outcome. Ubiquitination was identified initially as molecular tag which targets proteins for degradation (Ciechanover et al. 1980). The Lys48- linked polyubiquitination served as the signal for proteasomal degradation (Chau et al. 1989)(Avram Hershko and Ciechanover 1998). However, over the years, it has

been found that ubiquitin plays an important role in a wide variety of processes such as DNA repair, endocytosis, signalling and autophagy to name a few (Chen and Sun 2009).

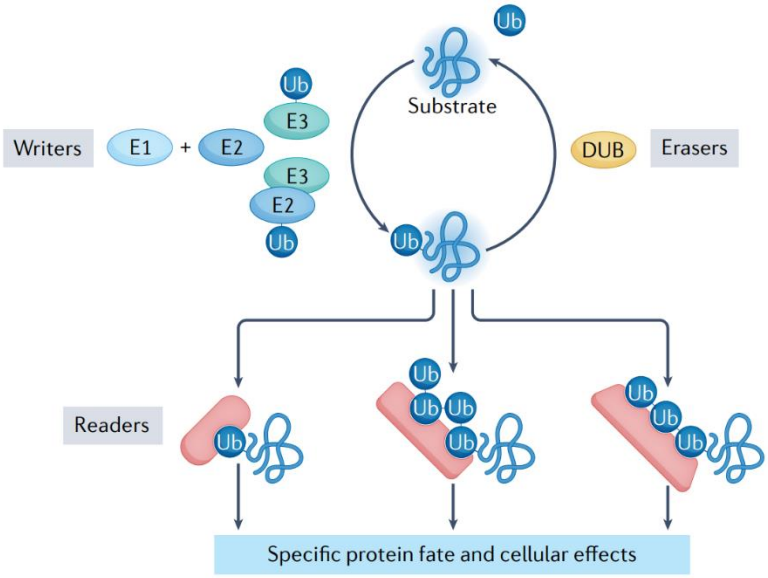


Figure 2.2.1: The Ubiquitin code.

E1,E2, and E3 enzymes act as 'writers' that ubiquitinates the target substrate. 'Readers' are the ubiquitin binding domains (UBD) containing proteins that elicit a specific biological outcome and DUBs are the 'erasers' that reverse the modification. These are the players that modify the ubiquitin code. Taken from reference (Dikic and Schulman 2022).

2.2.2 E1 activating enzymes

The ubiquitin activating enzymes initiate the process of ubiquitination. In yeast, there is only one E1 enzyme while humans have two. The mechanism of E1 catalysis is well understood (Ciechanover et al. 1980, 1982; Haas and Rose 1982; Pickart 2001). In the presence of ATP and Mg^{2+}, ubiquitin forms an adenylate intermediate that is strongly bound to the E1 with release of PPi. In the next step, the ubiquitin is transferred to the active site cysteine of E1 with the release of AMP. Then, one more molecule of ubiquitin adenylate is bound to E1 before it transfers the thiol linked ubiquitin to the cognate E2 enzyme. So, the activated E1 enzyme has two ubiquitin molecules loaded to it, one in the form of adenylated ubiquitin and other in the form of a thiol ester. The E1 is a very efficient enzyme which provides the bulk of activated ubiquitin required for all the downstream processes (Haas and Rose 1982).

2.2.3 E2 conjugating enzymes

Cells normally have one E1 enzyme, significant amount of E2 enzymes and hundreds of E3 ligases. E2 enzymes were initially thought as just ubiquitin carriers that transfers the ubiquitin to their

cognate ligases. But, in recent years, it has been increasingly understood that they play a very important role in the regulation of the ubiquitin code (Pickart 2001).

Humans contain around 40 E2s and yeast genome encodes around 13 E2s. All the E2s contain the ubiquitin conjugating (UBC) domain. This domain is roughly around 150 amino acids and consists of four standard helices, a short 3_{10} helix, and a four-stranded antiparallel β-sheet (Ye and Rape 2009).

E2 conjugating enzymes engage both with E1 and E3 enzymes. After E1 is activated with ubiquitin, conformational changes occur in E1 which exposes the binding site for E2 and the subsequent transfer of ubiquitin to the active site cysteine of the E2 forming a thioester linkage (D. T. Huang et al. 2007; Jin et al. 2007; I. Lee and Schindelin 2008). The E2s that modify other ubiquitin like proteins (UBLs) like SUMO and Nedd8 are very similar in structure compared to the one for ubiquitin. The specificity for a particular modifier arises because E2 interacts with strong affinity only to the activated E1. They bind weakly to free ubiquitin and to the free E1 enzyme (Haas, Bright, and Jackson 1988; A Hershko et al. 1983; Miura et al. 1999; Schulman and Wade Harper 2009).

Activated E2s then interact with E3 ligases to ubiquitinate the substrates. A single E2 can engage with one or more E3 ligases. The E3s generally recognize E2s through the L1 and L2 loops and the N-terminal α-helix 1 on the its surface (L. Huang et al. 1999)(Zheng et al. 2000). E2s remain mainly as E2∼Ub conjugates in cell until its cognate E3 activates it (Siepmann et al. 2003). E3 binds with a very low affinity with the E2s. This low affinity binding might be advantageous because E1 and E3 bind E2 on overlapping surface. So, after getting activated by E1 only, E2 can bind E3 (Eletr et al. 2005). For the assembly of ubiquitin chains, E2 must bind E3 several times.

E2s play a very important role in chain elongation and contribute to the processivity of the ubiquitin chain formation (Mikaela D. Stewart et al. 2016; Ye and Rape 2009). After a ubiquitin is attached to the lysine of the substrate, the decision to elongate the chain is taken most of the times by the E2. There are E2s which can only initiate a chain. In one of the examples, the APC/C complex uses Ubc4 to initiate the ubiquitination, then Ubc1 elongates Lys48 linked conjugates to degrade the target substrates (Rodrigo-Brenni and Morgan 2007).

2.2.3.1 Ubc4/UBE2D family of E2 conjugating enzymes

Ubc4/UBE2D is one of the largest family of E2 enzymes. These family of E2 proteins contains only the UBC domain and are the smallest members of the E2 family. In the in-vitro studies, they bind to a wide variety of E3 ligases and transfer ubiquitin to any lysine on the ubiquitin. This promiscuity makes the UBE2D proteins an attractive choice of E2 for in-vitro studies (Brzovic and Klevit 2006).

In budding yeast, Ubc4 and Ubc5 were identified initially for their role in degrading short lived and abnormal proteins (Seufert and Jentsch 1990). In yeast, it serves as the E2 for many E3 ligases

including Rsp5 (Nuber and Scheffner 1999), SCF complex (Kus et al. 2004) and APC/C complex (Rodrigo-Brenni and Morgan 2007) to name a few. In fission yeast, Ubc4 was identified for its role in regulating mitosis by degrading the mitotic cyclin, Cdc13 (Javerzat, Cranston, and Allshire 1996; Osaka et al. 1997; Seino et al. 2003). Ubc4 also regulates the nuclear protein quality control (Matsuo et al. 2011). Ubc4 functions with the E3 ligase Dsc1 for the activation of sterol regulatory element binding protein (SREBP) transcription factors (Raychaudhuri and Espenshade 2015; E. V. Stewart et al. 2011). It also function with Pub1, the homolog of Rsp5 to regulate the endocytosis of GPI-Anchored Protein Ecm33 (Fang et al. 2014).In human, UBE2D family proteins play a very important role in regulating receptor tyrosine kinases (RTKs) ,Hedgehog, TGFβ, NFκB pathway, and p53, whose dysregulation contributes to many neurodegenerative disorders and cancer (Roman-Trufero and Dillon 2022).

2.2.4 E3 ligases

The final enzyme in the cascade of ubiquitination is the E3 ligase. The E3s are the critical components of this cascade as they determine the efficiency and substrate specificity of the ubiquitination reaction in most cases. The E3 reaction involves the binding of cognate E2 and its substrate leading to the conjugation of ubiquitin to the substrate (Zheng and Shabek 2017). There are two major families of E3: RING-type E3s and HECT-type E3s.

2.2.4.1 RING E3

RING (really interesting new gene) E3s are the largest E3 family with around 600 predicted members in humans (Deshaies and Joazeiro 2009). These proteins have the characteristic RING domain or U-box fold catalytic domain. They function by acting as scaffold for positioning the E2 and substrate for ubiquitin transfer (Zheng et al. 2002).

The mechanism of RING E3s is well understood. Initial structural studies with uncharged E2 revealed a unique interface for E2-RING binding far from the E2 active site cysteine (Yin et al. 2009). But no conformational changes were observed in E2. Subsequent studies with charged E2 revealed that the binding of E3 reduces the dynamic nature of E2∼Ub and stabilizes it in a closed conformation (Plechanovová et al. 2012). This closed conformation favors the transfer the ubiquitin to the substrate.

2.2.4.2 HECT E3

HECT E3s are a small family of E3s (around 28 in human) that contains a conserved domain of around 350 amino acids (Rotin and Kumar 2009). It was first identified in human papilloma virus

(HPV) E6-associated protein (E6AP), hence the name HECT (homologous to E6-AP carboxy terminus) (Huibregtse et al. 1995).

The mechanism of action of HECT domain is distinct from RING E3s. In HECT E3, a transthioesterification reaction occurs which involves the transfer of ubiquitin from the active site cysteine of E2 to the cysteine of E3. Then, the ubiquitin is transferred to the substrate lysine (Huibregtse et al. 1995). HECT E3s have a modular structure: an N-terminal lobe which binds E2 and a C-terminal lobe which has the active site cysteine (L. Huang et al. 1999). One of the well-studied examples of HECT E3 ligases is the yeast Rsp5.

2.2.4.2.1 Role of a HECT E3 Rsp5 in different processes

Rsp5 family of proteins (Nedd4 in humans) contains an N-terminal C2 domain (binds lipids), two to four WW (Trp-Trp) domains (recognizes a PY motif: PPxY) and a catalytic C-terminal HECT domain(Figure 2.2.2) (Rotin and Kumar 2009). Rsp5 binds with Ubc4 to catalyze the formation of Lys63 linked ubiquitin chains (H. C. Kim and Huibregtse 2009).

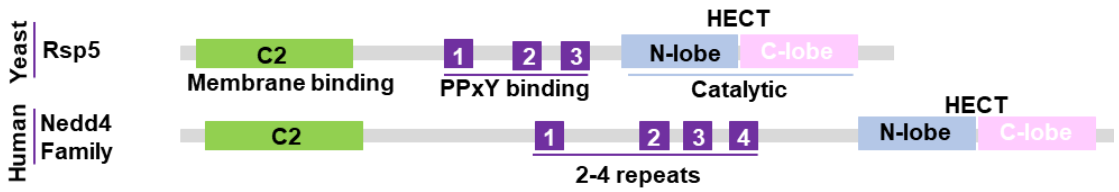


Figure 2.2.2: Domain architecture of Rsp5 family (Nedd4) of E3 ligases.

Rsp5 family of ligases bind their targets on the WW domains via interacting with the PY (PPxY) motif. Adapted from (Sardana and Emr 2021).

Rsp5 has been implicated to function in several cellular processes. It binds to the largest subunit of RNA polymerase and regulates transcription (Huibregtse, Yang, and Beaudenon 1997; Somesh et al. 2007). It is also important for proper mitochondrial inheritance (Fisk and Yaffe 1999). It is required for the proper export of mRNAs (Gwizdek et al. 2005; Rodriguez et al. 2003). It is also implicated in chromatin remodelling, tRNA and rRNA processing, regulation of fatty acid synthesis and combating various stresses (Kaliszewski and Żołądek 2008).

Rsp5 plays a very important role in the regulation of intracellular trafficking. It was initially identified for its role in the internalization of Gap1, a general amino acid permease in *Saccharomyces cerevisiae* (Hein et al. 1995). Subsequent studies showed that Rsp5 regulates the fate of almost all membrane transporters. As described earlier, it has been shown to regulate the endocytosis of several transporters in response to change in environmental conditions. Ubiquitination is the signal for

endocytosis which is mediated through the action of majorly one E3 ligase, Rsp5 (Dupré, Urban-Grimal, and Haguenauer-Tsapis 2004; Horák 2003).

Newly synthesized transporters from Golgi are sometimes directed to vacuoles for degradation depending on the nutrition conditions (Haguenauer-Tsapis and André 2004a)(Lauwers et al. 2010).This sorting also requires the ubiquitination by Rsp5. For example, newly synthesized siderophore transporter Sit1 is sorted to the vacuole via multivesicular body (MVB). Rsp5 ubiquitinates the transporter in the absence of its substrate FOB (ferrioxamine B) (Froissard et al. 2007). Thus, Rsp5 is the key ligase which controls the intracellular trafficking of different transporters.

The question then arises how a single enzyme can control the trafficking of so many different transporters? In yeast, not all transporters contain the PY motif which the WW domain of Rsp5/Nedd4 binds (Staub et al. 1996). To solve this problem, a group of proteins called arrestin-related trafficking adaptors (ART) or α-arrestin act as adaptors to mediate the ubiquitination of transporters (Léon and Haguenauer-Tsapis 2009). The α-arrestins contain the PY motif with which it interact with Rsp5 and brings it to close proximity to the transporter. They act as a bridge to make the E3 ligase come in proximity to the substrate. In yeast, there are around 14 α-arrestins identified till now (O'Donnell and Schmidt 2019). These proteins are activated in response to environmental stresses or signals and it has been shown that an individual arrestin can regulate many transporters (Nikko and Pelham 2009).

Rod1, Rog1 and Csr3 are the arrestins involved in the regulation of hexose transporters in yeast (Becuwe et al. 2012; Hovsepian et al. 2017; Nikko and Pelham 2009; O'Donnell and Schmidt 2019). In glucose rich conditions, these arrestins bind Rsp5 and ubiquitinate the transporters triggering their degradation. In low glucose conditions, activated AMPK phosphorylates these arrestins. Phosphorylation leads to binding of 14-3-3 which prevents its interaction with Rsp5 (Llopis-Torregrosa et al. 2016; O'Donnell and Schmidt 2019). Thus, Rsp5 E3 ligase with its cognate α-arrestins regulates the fate of variety of transporters.

2.2.5 Deubiquitinating enzymes (DUBs)

Deubiquitinating enzymes (DUBs) are the erasers of the ubiquitin code. They remove the ubiquitin chains from ubiquitinated proteins by cleaving the isopeptide bond or activate the ubiquitin precursors. DUBs are important regulators of ubiquitin system. They initiate the ubiquitin signaling by activating ubiquitin from precursors. Ubiquitin is always synthesized as precursor protein fused to ribosomal proteins or in the form of ubiquitin chains (Baker and Board 1987; Ozkaynak et al. 1987). DUBs cleave them so as to synthesize the active ubiquitin with the C-terminal diglycine motif. Second, they cleave the ubiquitin from the conjugated proteins thereby reversing the ubiquitination of the protein. Third, they trim the ubiquitin chains thereby modulating the downstream signaling. DUBs also

process ubiquitin chains cleaved from different proteins to maintain the ubiquitin homeostasis (Figure 2.2.3) (Reyes-Turcu, Ventii, and Wilkinson 2009). Most of the DUBs are cysteine-based proteases and possesses a catalytic dyad or triad consisting of cysteine that performs the nucleophilic attack, a histidine residue and a third acidic residue (Cstorer and Ménard 1994). DUBs also a possess a ubiquitin binding S1 site that bind ubiquitin. The hydrophobic patches on the ubiquitin Ile44 and Ile36 mediate the S1 binding, thereby contributing to the specificity for ubiquitin (Komander and Rape 2012).

There are around ~20 DUBs in yeast and more than 100 DUBs in humans. DUBs are classified into seven families based on their structure. Among them, the six family of cysteine proteases are the ubiquitin-specific proteases (USPs), the ovarian tumor proteases (OTUs), the ubiquitin C-terminal hydrolases (UCHs), the Josephin family, the motif interacting with ubiquitin (MINDY), and Zn finger and UFSP domain protein (ZUFSP/ZUP) family proteases. The JAB1/MPN/MOV34 metalloenzymes (JAMMs) are the Zn dependent metalloprotease family of DUBs (Kwasna et al. 2018; Mevissen and Komander 2017). One of the largest family of DUBs is the USP family. USP7 is one of the well-known members of this family of proteases.

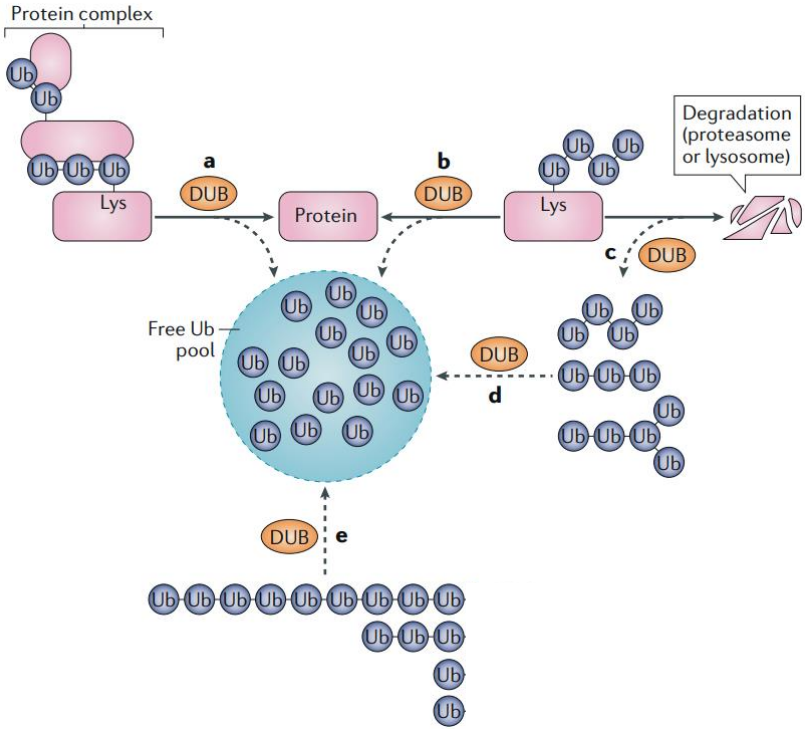


Figure 2.2.3: Role of DUBs in the ubiquitin system.

Deubiquitinating enzymes have mulitple roles in regulating the ubiquitin homeostasis in the cell. (a) they regulate signalling by removing regulatory ubiquitin (b) they protect proteins from proteasomal or lysosomal degradation (c) and (d) they recycle the ubiquitin (e) they activate the ubiquitin from genes coding ubiquitin-ribosomal fusion proteins or precursor proteins. Taken from reference (Komander, Clague, and Urbé 2009).

2.2.5.1 Mechanism and function of USP7

USP7 also known as Herpes virus associated protease (HAUSP) plays an important role in various cellular processes like DNA repair, gene expression, and immune response (Pozhidaeva and Bezsonova 2019). USP7 is a potent oncogene which is dysregulated in multiple cancers. It regulates the level of the tumour suppressor gene, p53 (Li et al. 2002).

USP7 is a modular DUB. It consists of a N-terminal TRAF-like (Tumor necrosis factor Receptor–Associated Factor) domain, the catalytic core domain and the five C-terminal ubiquitin-like domains, UBL1-5 (Holowaty et al. 2003). The TRAF-like domain binds the substrates of USP7. p53 was the first identified substrate which binds the TRAF-like domain (Hu et al. 2002). Many other substrates were identified in subsequent studies. The catalytic core of USP7 comprises of Fingers, Palm, and Thumb domain architecture. The catalytic triad is placed between the Palm and the Thumb and the ubiquitin binding happens in the Fingers (Hu et al. 2002). The catalytic mechanism is similar to that of the cysteine proteases of the papain family (Figure 2.2.4) (Cstorer and Ménard 1994). The UBL domain of USP7 also serves as a substrate binding site. A viral E3 ubiquitin ligase ICP0 binds to the UBL domains of USP7 as the ligase autoubiquitinates itself and is prone to degradation by proteasome. This is the first characterized substrate binding to the UBL of USP7 (Pfoh et al. 2015). Subsequent studies have characterized additional substrates of the UBL domain of USP7 (Faesen, Luna-Vargas, and Sixma 2012).

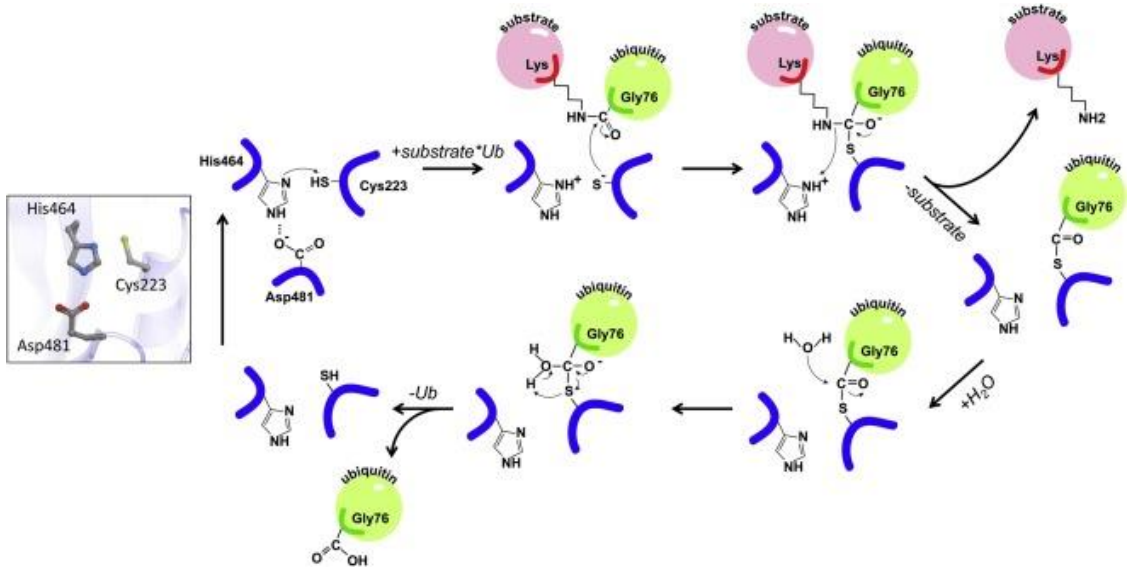


Figure 2.2.4: Mechanism of Usp7 catalysis

The C223, H464 and D481 of Usp7 form the catalytic triad of USP7. The nucleophilic attack of the isopeptide bond is carried out by C223 of the catalytic triad. H464 and D481 are part of the His box that acts as a proton acceptor. Taken from reference (Pozhidaeva and Bezsonova 2019).

2.2.6 Regulation of DUBs

As DUBs are important enzymes fine-tuning all the ubiquitin-dependent processes, regulation of DUBs becomes important for the proper functioning of cells. Dysregulation of DUBs can lead to a variety of disease conditions which includes cancer, inflammatory disorders, and neurogenerative diseases (Mevissen and Komander 2017). In general, DUBs can be regulated by altering its activity, its binding partners or its localization (Sahtoe and Sixma 2015).

Ubiquitin binding to the DUB can activate it. USP7 exists in an inactive state. Binding with the substrate rearranges the catalytic triad making it competent for the catalysis. The binding of linear Met1-linked ubiquitin chains relieves the inhibitory conformation and aligns the catalytic triad of the DUB, OTULIN for processing (Keusekotten et al. 2013). Most of the DUBs are modular in nature. In USP7, the last two UBL domains along with C-terminal peptide enhances the activity of the enzyme by stabilizing the catalytic conformation of the enzyme (Rougé et al. 2016). Also, many of the DUBs attain their optimal activity only when they are part of multi-protein complexes. The proteasomal DUBs, RPN11, USP14, and UCHL5 are activated only when they are part of the proteasome complex (de Poot, Tian, and Finley 2017). External proteins also can affect the activity of DUBs. One interesting example is the UbcH5B mediated stimulation of Lys48-linked polyubiquitin hydrolysis by OTUB1. UbcH5B is a E2 conjugating enzyme that enhances the activity of OTUB1 by stabilizing a ubiquitin binding site in it (Wiener et al. 2013).

DUBs can be regulated by posttranslational modifications (PTM) also. The deubiquitinase OTUD5 is phosphorylated at Ser177 and phosphorylation is necessary to activate the enzyme. It does so by stabilizing the substrate binding (O. W. Huang et al. 2012) DUBs can themselves be ubiquitinated. Monoubiquitination of UCH-L1 near its active site, restrict its enzyme activity. Interestingly UCH-L1 itself can self-deubiquitinate the ubiquitin conjugate, thereby reversing the inhibition (Meray and Lansbury 2007).So, wide range of posttranslational modifications regulate the activity of different DUBs (Das et al. 2020).

Systematic analysis of the subcellular localization of DUBs in fission yeast and human revealed the presence of DUBs in diverse cellular compartments (Kouranti et al. 2010; Urbé et al. 2012). The localization of DUBs also changes during different conditions. During DNA damage, ATM-mediated phosphorylation of USP10 leads to nuclear translocation and stabilization of p53 (Yuan et al. 2010). Caesin kinase 2 phosphorylates OTUB1 leading to its nuclear accumulation during DNA repair (Herhaus et al. 2015). Inversely, phosphorylation of USP4 by Akt leads to its relocation to cytosol from nucleus to deubiquitinate TGF-beta type I receptor (L. Zhang et al. 2012). Thus, DUBs can be regulated by altering its localization.

2.2.7 Ubiquitin and membrane trafficking

One of the first non-proteasomal function of ubiquitination was its role in intracellular trafficking (Hicke and Riezman 1996). Since then, the role of ubiquitination in all the aspects of the trafficking are well studied (Berlin et al. 2023; Haglund and Dikic 2012).

The role of ubiquitination in internalization was first demonstrated for the transporter, Ste2p, in yeast. Ubiquitination acted as the signal which triggered the endocytosis of the transporter and subsequent degradation in vacuole (Hicke and Riezman 1996). Subsequent studies has demonstrated the importance of ubiquitination for endocytosis in both humans and yeast (MacGurn, Hsu, and Emr 2012). After ubiquitination, the proteins containing ubiquitin-interacting motif(UIM) domains interact with the cargo and sort it into the endocytic vesicles (MacGurn, Hsu, and Emr 2012).

After internalization, the fate of the transporters is decided at the endosomes. Endosomes are organelles derived either from the plasma membrane or the Golgi. Proteins are either recycled back to plasma membrane or degraded via the lysosome/vacuole. After endocytosis, multivesicular body (MVB) is formed which fuses with the vacuole (Lauwers et al. 2010). MVB formation is mediated by the evolutionarily conserved ESCRT (endosomal sorting complex required for transport) complex (Henne, Buchkovich, and Emr 2011). The ESCRT-0 comprises of two proteins containing the ubiquitin-binding domains (UBD): Vps27 and Hse1 in yeast. They bind the ubiquitinated cargo and initiate the MVB formation. Subsequent interaction with the other components of ESCRT complex completes the MVB formation.

The cargo sorting at the MVB is regulated by various DUBs. AMSH and USP8 both bind to the Src homology 3 (SH3) domain of the ESCRT-0 protein STAM (Hse1 in yeast). These DUBs play an important role in regulating EGFR trafficking and degradation in humans (Clague, Liu, and Urbé 2012). In yeast, Doa4 is recruited to Snf1, an ESCRT-III component via binding to Bro1 (Dupré and Haguenauer-Tsapis 2001; Luhtala and Odorizzi 2004). It recycles the ubiquitin from the cargoes before degradation, thereby playing an important role in ubiquitin homeostasis (Swaminathan, Amerik, and Hochstrasser 1999). In humans, UBPY function similar to Doa4 (Mizuno et al. 2006).

For cargo sorting from Golgi to endosomes, ubiquitin plays an important role. Carboxypeptidase S (Cps1), a vacuolar hydrolase in budding yeast is ubiquitinated by Rsp5 and it is sorted to the vacuole via the MVB pathway (Katzmann, Babst, and Emr 2001). Many transporters are sorted directly from Golgi to vacuole via endosome in conditions which can trigger their endocytosis. Rsp5 is the E3 ligase that ubiquitinates the cargoes at the Golgi (Schwabl and Teis 2022). At the Golgi, γ-ear-containing ARF-binding proteins or GGAs sort the cargoes from the Golgi to endosome. They bind the ubiquitinated cargoes and sort them to endosomes (Puertollano et al. 2001; Puertollano and Bonifacino 2004). Thus, ubiquitin plays an important role in regulating all aspects of intracellular trafficking.

2.2.8 Ubiquitin and Nutrient stresses

Glucose act as the fundamental source of nutrient as well as a signaling molecule in all eukaryotic cells (Rolland, Winderickx, and Thevelein 2001; Towle 2005). Therefore, cells have evolved elaborate mechanism for the uptake and utilization of glucose. In yeast, the uptake of glucose is mediated by the expression of glucose transporters (*HXTs*) (Sabire and Mark 1999). In the absence of glucose, Rgt1 represses the expression of the *HXTs* by binding in the promoters of these genes. Mth1 and Std1 act as co-repressors that facilitates the binding of Rgt1 to *HXT* promoters. Glucose is sensed through the presence of two glucose sensors/receptors, Rgt2 and Snf3 (Ozcan et al. 1996). These plasma membrane proteins are activated by different concentrations of glucose. In the presence of high glucose, Rgt2 is activated and leads to the expression of low-affinity glucose transporters like Hxt1 and Hxt3. Whereas activation of Snf3 by low levels of glucose leads to the expression of high-affinity glucose transporters like Hxt2 and Hxt4 (Schmidt et al. 1999). These glucose sensors activate membrane-associated protein kinase casein kinase I (Yck1) which phosphorylates Mth1 and Std1 (Moriya and Johnston 2004). This phosphorylation leads to the ubiquitination of Mth1 and Std1 by SCF^{Grr1} ubiquitin-protein ligase and leads to its proteasomal degradation (Flick et al. 2003; Jeong-Ho et al. 2006). Snf1, the yeast homolog of mammalian AMP-activated protein kinase (AMPK), also represses the expression of *MTH1* (Aneta et al. 2004). The reduction in the level of Mth1 leads to the phosphorylation of Rgt1 by the cAMP-dependent protein kinase A (PKA). This leads to the dissociation of Rgt1 from the promoters of *HXTs*, thereby inducing the expression of the glucose transporters (J.-H. Kim and Johnston 2006; Palomino, Herrero, and Moreno 2006). Thus, the expression of *HXT* genes is tightly regulated to ensure the proper survival of cells in different glucose conditions (J.-H. Kim et al. 2013).

The glucose transporters are also posttranslationally regulated based on the glucose conditions, During high glucose conditions, high-affinity glucose transporters such as Hxt6 and Hxt7 are endocytosed and degraded in the vacuole (Horák 2003; Krampe et al. 1998). The low-affinity transporters such as Hxt3 and Hxt1 are endocytosed and degraded in glucose-limiting conditions (Roy et al. 2014; Snowdon et al. 2009). Both these processes are mediated by the ubiquitination of the glucose transporter. This ubiquitination is carried out by the Nedd4-like E3 ubiquitin ligase Rsp5 (MacGurn, Hsu, and Emr 2012). The details regarding the mechanism and regulation of Rsp5 is described in section 2.2.4.2.1.

Nitrogen is also one of the most important elements of metabolism as it is an essential building block of nucleic acids and amino acids (Rødkær and Færgeman 2014). During nitrogen starvation, cells undergo changes in transcription, translation and protein degradation pathways. Yeast cells react to the changes in the amount and quality of the nitrogen source. Glutamine is the preferred nitrogen source for lab growing yeast strains (Zaman et al. 2008). During nitrogen-poor conditions, Gln3 and Gat1, two GATA-type zinc finger transcription factors, induce the expression of genes involved in combating the nitrogen stress. The expression of amino acid permeases is upregulated during nitrogen starved conditions by the same transcription factors (Stanbrough and Magasanik 1995). The availability of the

nitrogen is sensed via the TOR signaling (Wullschleger, Loewith, and Hall 2006). During nitrogen-rich conditions, Gln3 and Gat1 are phosphorylated by the TOR complex which sequesters it in the cytoplasm, thereby repressing the transcription of genes involved in combating nitrogen stress such as amino acid permeases (Beck and Hall 1999). The TOR signaling also has been to shown to regulate the amino acid permeases. In the presence of nitrogen, TORC1 complex phosphorylates Npr1 which inactivates it. Active Npr1 inhibits the activity of Art1, an arrestin-like adaptor protein that facilitates the endocytosis and degradation of specific amino acid permeases. Thus, TORC1 complex promotes the endocytosis of amino acid permeases in nitrogen-rich conditions (MacGurn et al. 2011).

Ubiquitination plays an important role in regulating the amino acid permeases. Gap1, the general amino acid permease, is ubiquitinated by the E3 ligase Rsp5 and its adaptors, Bul1 and Bul2 in the presence of nitrogen (Helliwell, Losko, and Kaiser 2001). The ubiquitination leads to the endocytosis and its subsequent degradation in vacuole. Interestingly, high affinity tryptophan permease, Tat2 is degraded in the vacuole in the nitrogen starved conditions. This is also mediated by the process of ubiquitination (Beck, Schmidt, and Hall 1999). Thus, ubiquitination plays a key role in combating nitrogen stress.

2.2.9 Protein Myristoylation

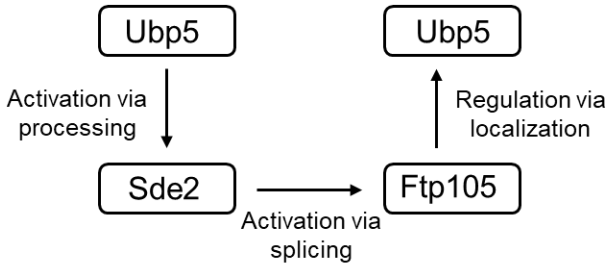
N-myristoylation is one of the major lipid modifications in the cell in which myristic acid is added to the N-terminal glycine of a protein (Boutin 1997; Farazi, Waksman, and Gordon 2001). Myristoylation has been shown to be involved in signal transduction, protein stability, and in the localization of the protein (Wright et al. 2010). N-myristoylation is carried out by the enzyme called N-myristoyltransferase (NMT) (Towler et al. 1987). It generally occurs cotranslationally but there are evidences of posttranslational myristoylation as well (Martin, Beauchamp, and Berthiaume 2011). During cotranslational protein N-myristoylation, initiator methionine is removed by the action of methionine aminopeptidase and then NMT ligates myristic acid to the exposed glycine. Amino acid sequence of most of the NMT substrates are similar and has either serine or threonine at fifth position (G-X-X-X-X-S/T-X-X-X-X) (Rudnick et al. 1991). Myristoylation was considered to be an irreversible process. However, it has been recently shown that invasion plasmid antigen J (IpaJ) protein secreted by *Shigella flexneri* has a demyrisloylating activity (Burnaevskiy et al. 2013).

One of the important functions of myristoylation modification is promoting the binding of protein to the cell membrane. However, myristoylation is not sufficient for the binding of protein to the membrane. There are evidences which suggest the presence of an extra signal which ensures the stable binding of the myristoylated protein to the membrane (Resh 1999). These extra signals can be a polybasic cluster of amino acid like in Src, HIV-1 Gag or a palmitate moiety as observed in STK16(Ser/Thr kinase 16) (Sigal et al. 1994; Junjun Wang et al. 2019).

2.3 PRELUDE TO THE STUDY

Eukaryotic cell sorts proteins of diverse functions into their distinct subcellular compartments. One of the pathways for sorting of proteins is the secretory pathways. Secretory pathway comprises of the proteins synthesized in the Endoplasmic reticulum (ER). After undergoing modifications in the ER, the proteins enter the Golgi apparatus which sorts the proteins to different compartments (Guo, Sirkis, and Schekman 2014). To ensure accurate sorting of proteins, cells employ variety of protein quality control pathways (Schwabl and Teis 2022).

Ubiquitination of proteins act as an important signal to regulate the fate of proteins. Ubiquitin is a highly conserved modifier that is conjugated to different proteins to elicit a particular outcome. Ubiquitination is brought about by the activity of three enzymes: E1 activating enzyme, E2 conjugating enzyme, and E3 ubiquitin ligase. These enzymes act as the writers of the ubiquitin code. Like phosphorylation, ubiquitination is a reversible modification. The erasers of the ubiquitin code are the deubiquitinating enzymes (DUBs) that cleave ubiquitin from the proteins (Pickart 2001). Although several E3 ligases have been identified and characterized at the Golgi, the role of DUBs is not well explored (Schwabl and Teis 2022). Systematic analysis of subcellular localization of 20 DUBs in *Schizosaccharomyces pombe* revealed Ubp5 as the only DUB which is localized at the Golgi. Ftp105, a Golgi anchored protein, localizes Ubp5 to the Golgi (Kouranti et al. 2010). The function of Ubp5 at Golgi is not known.



Schematic 1: Summary of interaction between Sde2, Ubp5 and Ftp105. The Sde2 precursor is processed by Ubp5/Ubp15 which leads to the generation of Sde2-C. Sde2-C is in turn required for the proper splicing of *ftp105-i3*. Ftp105 regulates the Golgi localization of Ubp5.

In *S. pombe*, Ubp5 along with its paralog Ubp15, activate the ubiquitin-fold containing splicing regulator Sde2 by processing it in the invariant GG–K motif (Thakran et al. 2018). In the previous chapter, I showed that Ubp5 along with Ubp15 activates Sde2 which is required for its splicing activity. And Sde2 is required for the proper splicing of Ftp105 (Schematic 1). So, we were interested in studying the function of Ftp105 and Ubp5.

Ubp5 along with four other DUBs regulate endocytosis and cell polarity in *S. pombe* (Kouranti et al. 2010). In *Saccharomyces cerevisiae*, Ubp15, the homolog of Ubp5, controls the timing of entry

of S phase by controlling the stability of Clb5, a S phase cyclin (Ostapenko, Burton, and Solomon 2015). Ubp15 is associated with the peroxisomal export machinery and deubiquitinate the Pex5p receptor (Debelyy et al. 2011; Schwerter et al. 2018). Interestingly, Ubp15 also positively regulates endocytosis by preventing the degradation of arrestin-related trafficking adaptors (ARTs) (Ho, MacGurn, and Emr 2017). Recently it has been shown that Ubp15 promotes the nuclear export of mRNAs by deubiquitinating the main nuclear export receptor Mex67 (Eyboulet et al. 2020). Homolog of Ubp5, USP7 plays an important role in diverse cellular processes including DNA damage response, transcription, immune response to name a few. USP7 overexpression leads to cancer as it decreases the level of the tumor suppressor p53 (Pozhidaeva and Bezsonova 2019). The nuclear function of USP7 is well characterized. However, the role of USP7 in sorting cargoes at the Golgi is not known.

In *Caenorhabditis elegans*, HID-1, the homolog of Ftp105, was identified in a screen for mutants showing constitutive Dauer formation at 27ºC. Dauer is an arrested third stage larvae in *C. elegans* which it forms when it gets exposed to adverse environmental conditions (Cassada and Russell 1975). One of the conditions which induces Dauer formation is high temperature (Golden and Riddle 1984). Genetic analysis has revealed two classes of dauer mutants. Dauer formation constitutive (Daf-c) which forms dauers in favourable conditions and Dauer formation defective (Daf-d) which fails to form dauer in inducing conditions (Vowels and Thomas 1992). Daf-c mutants were initially screened at 25°C (Malone and Thomas 1994). To identify more mutants, a screen was performed at 27ºC and HID-1 (high-temperature-induced dauer formation) was identified (Ailion and Thomas 2003). It was shown to act in the insulin branch of dauer formation. HID-1 plays an important role in neuropeptide sorting and secretion signaling by controlling the formation of Large Dense Core Vesicles (LDCVs). Loss of HID-1 leads to the mis-sorting of cargoes to lysosome (Mesa et al. 2011; Yu et al. 2011). LDCVs are specialized vesicles that stores cargoes such as peptide hormones or neuropeptides in secretory cells (Tooze and Huttner 1990). In pancreatic cells, the loss of HID-1 leads to defective insulin secretion. It plays an important role in homotypic fusion of immature secretory granules (ISG) that is essential for proinsulin processing (Du et al. 2016). In rat endocrine cells, HID-1 regulates the sorting of neuropeptides and peptide hormones by controlling trans-Golgi network (TGN) acidification. Loss of HID-1 leads to the mislocalization of a Golgi-enriched vacuolar H+ ATPase subunit (Hummer et al. 2017). Interestingly, HID-1 is a peripheral membrane protein localized primarily in medial and trans Golgi network through N-terminal myristoylation (L. Wang et al. 2011).

Although much is known about the function of HID-1/Ftp105 in vesicular trafficking, the significance of its interaction with Ubp5 is not clear.

2.4 RESULTS

2.4.1 N-terminal myristoylation is critical for the Golgi localization and function of Ftp105

HID-1, the mammalian homolog of Ftp105, is localized to the Golgi via a N-terminal myristoylation modification (L. Wang et al. 2011). Multiple sequence alignment of the putative orthologs of Ftp105 showed conservation of the 2nd position N-terminal glycine which gets myristoylated across species (Figure 2.4.1A). So, we wanted to test whether N-terminal myristoylation is required for the Golgi localization of Ftp105 in *Schizosaccharomyces pombe*. We chromosomally tagged Vrg4 with a C-terminal mCherry to act as a Golgi marker. Vrg4 is a transmembrane protein involved in the transport of GDP-mannose into early Golgi (Losev et al. 2006).We made Ftp105 tagged constructs with C-terminal GFP expressed under *ftp105*'s own promoter. We also made a myristoylation defective Ftp105 construct where the 2nd glycine was mutated into alanine(G2A) We transformed both the constructs in the Vrg4 tagged strains and performed microscopy to visualize the co-localization of Ftp105 and Vrg4 (Figure 2.4.1B). Wildtype Ftp105 colocalized with Golgi marker Vrg4 whereas the myristoylation defective mutant did not, indicating that the myristoylation modification is necessary for the proper localization of Ftp105 to Golgi.

The deletion of Ftp105 showed growth defects in high and low temperatures. We then did complementation assays to check if Golgi localization is important for the function of Ftp105. We transformed the wildtype and the myristoylation defective(G2A) construct in *Δftp105* strain. The mutation of glycine (G2A) did not complement the growth defects of *Δftp105* strain (Figure 2.4.1C). Hence, Ftp105 localizes to Golgi via its N-terminal myristoylation modification and the proper localization of Ftp105 to Golgi is required for its activity.

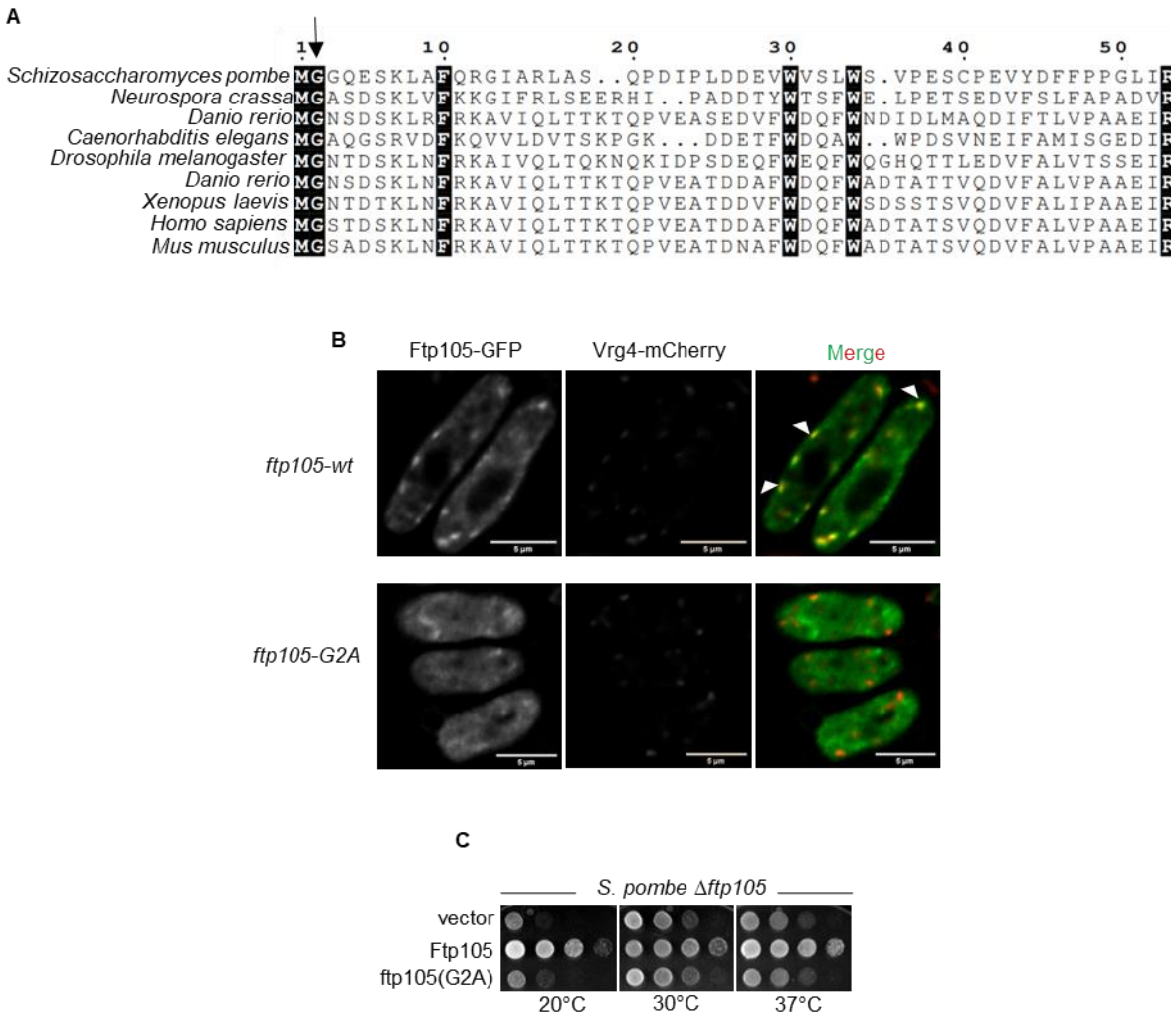


Figure 2.4.1: The myristoylation defective Ftp105 is not functional and does not localize to the Golgi.

A) Multiple sequence alignment of first ~50 amino acids the putative orthologs of Ftp105. B) Cells expressing wildtype (wt) and myristoylation defective (G2A) Ftp105 with GFP tag in Vrg4 tagged strain is imaged for localization of Ftp105-GFP with Vrg4-mCherry. Arrows indicate the colocalization of Ftp105 with Vrg4. Scale bar = 5µm. C) Complementation of *S. pombe Δftp105* by wildtype and myristoylation defective(G2A) Ftp105. All constructs were expressed from Ftp105's own promoter. Five-fold serial dilution spotting was done on indicated agar plates. Plates were incubated at the indicated temperatures until growth appeared.

2.4.2 Ftp105 is required for the Golgi localization of Ubp5

It has been shown that Ubp5 localizes to Golgi and this localization depends on Ftp105 (Kouranti et al. 2010).To verify this, we chromosomally tagged Ubp5 and Vrg4 with a C-terimnal GFP and mCherry tags respectively in wildtype strain. Upon microscopic analysis, we observed that Ubp5 colocalize with the Golgi marker Vrg4 (Figure 2.4.2A). In the absence of Ftp105, Ubp5 is diffused in the cytosol (Figure 2.4.2B). This indicates that Ftp105 is required for the Golgi localization of Ubp5.

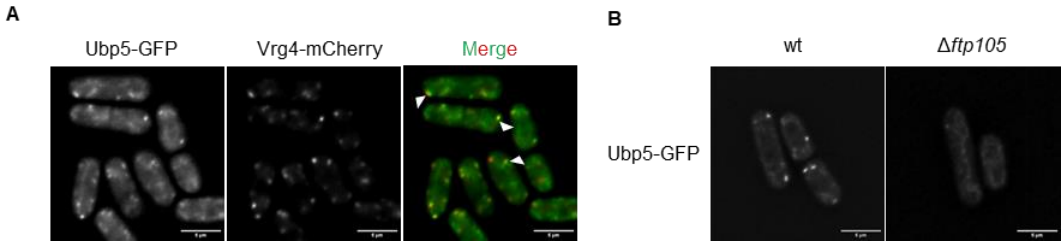


Figure 2.4.2: Ubp5 localizes to the Golgi in a process dependent on Ftp105.

A) Ubp5 and Vrg4 were chromosomally tagged with a C-terminal GFP and mCherry tags respectively in wildtype strain. The strains were grown in EMM minimal media and imaged. Scale bar = 5µm. B) Wildtype and *Δftp105* strains with chromosomally tagged Ubp5 was imaged. Scale bar = 5µm.

2.4.3 Ftp105 forms a complex with the DUB Ubp5

We observed that Ftp105 is essential for the recruitment of Ubp5 to Golgi. It has also been shown that Ftp105 and Ubp5 colocalize with each other (Kouranti et al. 2010). We then asked the question whether they are also part of the same complex in the cell? To address this, we made chromosomal tagged strains of Ftp105 and Ubp5 with C-terminal 6HA epitope tag. We then performed immunoprecipitation to purify the complexes using anti-HA beads. The purified complex was subjected to mass spectrometric analysis to identify the co-immunoprecipitated proteins. Indeed, Ftp105 co-purified with Ubp5 and Ubp5 co-purified with Ftp105 (Figure 2.4.3A). To verify the mass spectrometric analysis, we transformed Ftp105 constructs with 3FLAG epitope in Ubp5 chromosomally tagged strains with C-terminal 6HA and immunoprecipitated Ftp105 and checked for Ubp5. We observed that Ubp5 co-immunoprecipitates with Ftp105 (Figure 2.4.3B). These results indicate that Ftp105 and Ubp5 are part of the same complex.

We then performed two hybrid assays to see if there is interaction between Ftp105 and Ubp5. Indeed, we observed that Ftp105 and Ubp5 interact with each other in the two hybrid assays (Figure 2.4.3C). Thus, Ftp105 and Ubp5 are part of the same complex in the cell.

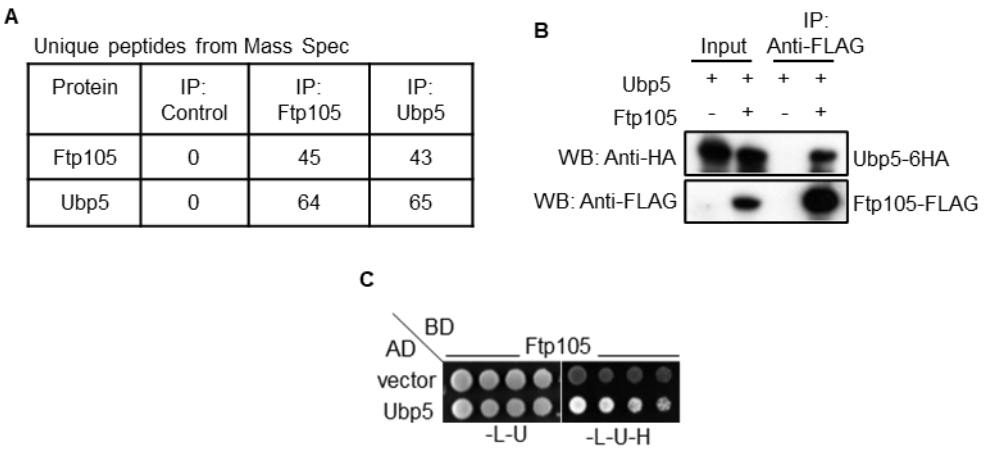


Figure 2.4.3: Ftp105 and Ubp5 are part of the same complex.

A) Lysates from cells expressing chromosomally tagged Ftp105-6HA and Ubp5-6HA respectively was immunoprecipitated using anti-HA beads. The immunoprecipitated complexes were analyzed using mass spectrometry. The table represents the unique peptides obtained for the specified protein. B) Wildtype strain with Ubp5-6HA tagged is transformed with constructs expressing Ftp105-3FLAG under its own promoter. Immunoprecipitation was performed with anti-FLAG beads and were analyzed by western blot analysis. C) In yeast two-hybrid assay pGBDU1-Ftp105 and pGADC1-Ubp5 were co-transformed, and transformants were monitored for interaction on selective plates.

2.4.4 Ubp5 is epistatic over Ftp105

Ftp105 is required for the Golgi localization of Ubp5 and they are part of the same complex in the cell. To further understand the functioning of Ftp105 and Ubp5, we assessed the genetic interactions between Ftp105 and Ubp5. We made double deletions of Ftp105 and Ubp5 and that of Ftp105 and Ubp15, the paralog of Ubp5 in *S. pombe* and checked their phenotype (Figure 2.4.4A). Interestingly, *Δftp105 Δubp5* did show more severe phenotype than *Δftp105* and shows a defect similar to the deletion of Ubp5. But *Δftp105 Δubp15* showed severe growth phenotype compared to the single mutants at high and low temperatures. Similarly, it has been previously shown that *Δubp5 Δubp15* strain shows severe growth defect compared to the single mutants (Thakran et al. 2018). This suggests that Ftp105 and Ubp5 function in the same pathway and Ubp5 is epistatic over Ftp105. Ftp105 and Ubp15 showed a negative genetic interaction and might function in different pathways, or they are redundant to each other.

We also observed that the overexpression of Ftp105 is toxic in wildtype cells (Figure 2.4.4B). We speculated that the overexpression of Ftp105 could lead to sequestration of Ubp5 leading to growth defects. To test this, we also tested the toxicity of Ftp105 overexpression in *Δubp5* strain. Ftp105 overexpression was not toxic in *Δubp5* (Figure 2.4.4B). On the contrary, it rescued the growth defects in *Δubp5* at 20ºC. Thus, the toxicity of Ftp105 was mediated through its action on Ubp5. This corroborated the findings that Ftp105 and Ubp5 act in the same pathway and Ftp105 functions via its interaction with Ubp5.

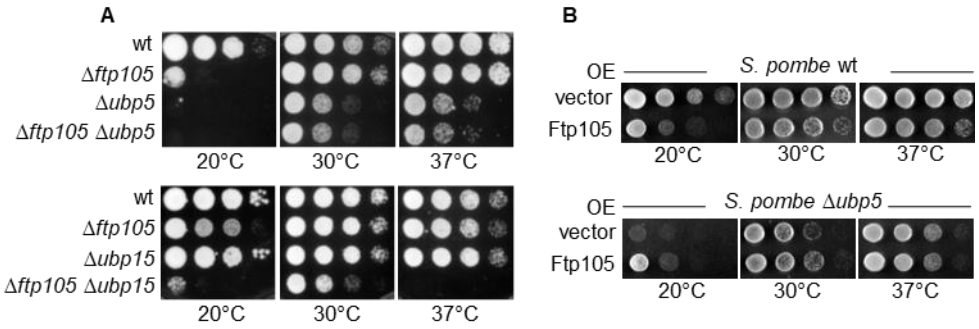


Figure 2.4.4: Ftp105 and Ubp5 act in the same pathway.

A) Genetic interaction between Ftp105 and Ubp5 and between Ftp105 and Ubp5. Five-fold serial dilution spotting of indicated mutant strains was done on rich media. Plates were incubated at the indicated temperatures until growth appeared. B) Overexpression of Ftp105 in the indicated strains grown at different temperatures. Ftp105 was expressed under a strong *eno101* promoter.

2.4.5 The Ftp105-Ubp5 complex may combat nutritional stresses

To identify the processes affected in the loss of Ftp105 and Ubp5, we performed growth assays of *ftp10*5 and *ubp5* deletion strains in different stress conditions. We observed that Ftp105 and Ubp5 deletion strains grow slower in synthetic media (Figure 2.4.5A). Cells grown in low nitrogen induce expression of amino acid permeases that are trafficked to the plasma membrane and rescue the growth defects observed in synthetic media (Weisman et al. 2005). Therefore, we monitored growth of *ftp10*5 and *ubp5* deletion strains in low nitrogen (ammonium sulphate conc.= 0.5g/l) and nitrogen-replete media (ammonium sulphate conc.= 5g/l). Growth defects in *ftp10*5 and *ubp5* deletion strains were rescued in low-nitrogen media (Figure 2.4.5A). This suggests that Ftp105 and Ubp5 could regulate the localization of amino acid permeases at the plasma membrane. On the other hand, the absence of Ftp105 and Ubp5 led to slow growth in low glucose media (Figure 2.4.5B) possibly because of insufficient glucose transporters in the plasma membrane.

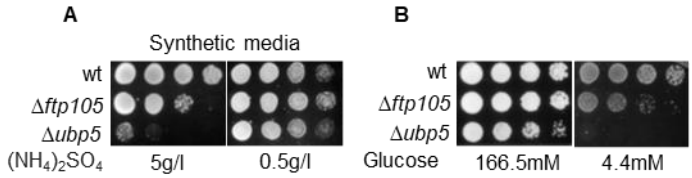


Figure 2.4.5: Ftp105 and Ubp5 may combat nutritional stresses.

A) Growth assay of wildtype(wt), *Δftp105* and *Δubp5* strains in EMM minimal media with control nitrogen (ammonium sulphate conc.= 5g/l) and low nitrogen (ammonium sulphate conc.= 0.5g/l) conditions. B) Growth assay of wildtype(wt), *Δftp105* and *Δubp5* strains in media with 166.5mM (3%) glucose and 4.4mM (0.08%) glucose.

2.4.6 Ubiquitinated proteome of Ftp105 and Ubp5

Earlier we hypothesized that Ftp105-Ubp5 complex may deubiquitinate transporters involved in alleviating nutritional stresses. To confirm the hypothesis, we identified ubiquitinated targets of Ftp105 and Ubp5. We integrated a ubiquitin construct with a N-terminal 6HIS tag expressed under a strong *eno101* promoter in wildtype (wt), *Δftp105,* and *Δubp5* strains and performed denaturing Ni-NTA pulldown assays and analyzed the proteins using mass spectrometry. Ftp105 and Ubp5 shared many substrates (Figure 2.4.6A). We verified the mass spectrometry data by checking the ubiquitination status of one of the substrates, Ubc13 (Figure 2.4.6B). We then looked into substrates that were either membrane proteins or proteins that were known to play a role in vesicular trafficking as we hypothesized that Ftp105 and Ubp5 might regulate the localization of membrane transporters. We found many such substrates which included plasma membrane transporters, e.g., Ght5 and Pho84, ER membrane proteins, and arrestin-related trafficking adaptors (ARTs) (Figure 2.4.6C). ARTs are positive regulators of endocytosis and degradation of transporters. ARTs bind and get ubiquitinated by the E3 ligase Rsp5/Pub1. The activated arrestin-E3 ligase complex then ubiquitinates plasma membrane transporters triggering their endocytosis (Barata-Antunes et al. 2021). We also found many cytoplasmic and nuclear proteins as the substrates of Ftp105 and Ubp5. Thus, the Ftp105-Ubp5 complex appear to deubiquitinate a wide variety of substrates and many of them are part of the plasma membrane proteome.

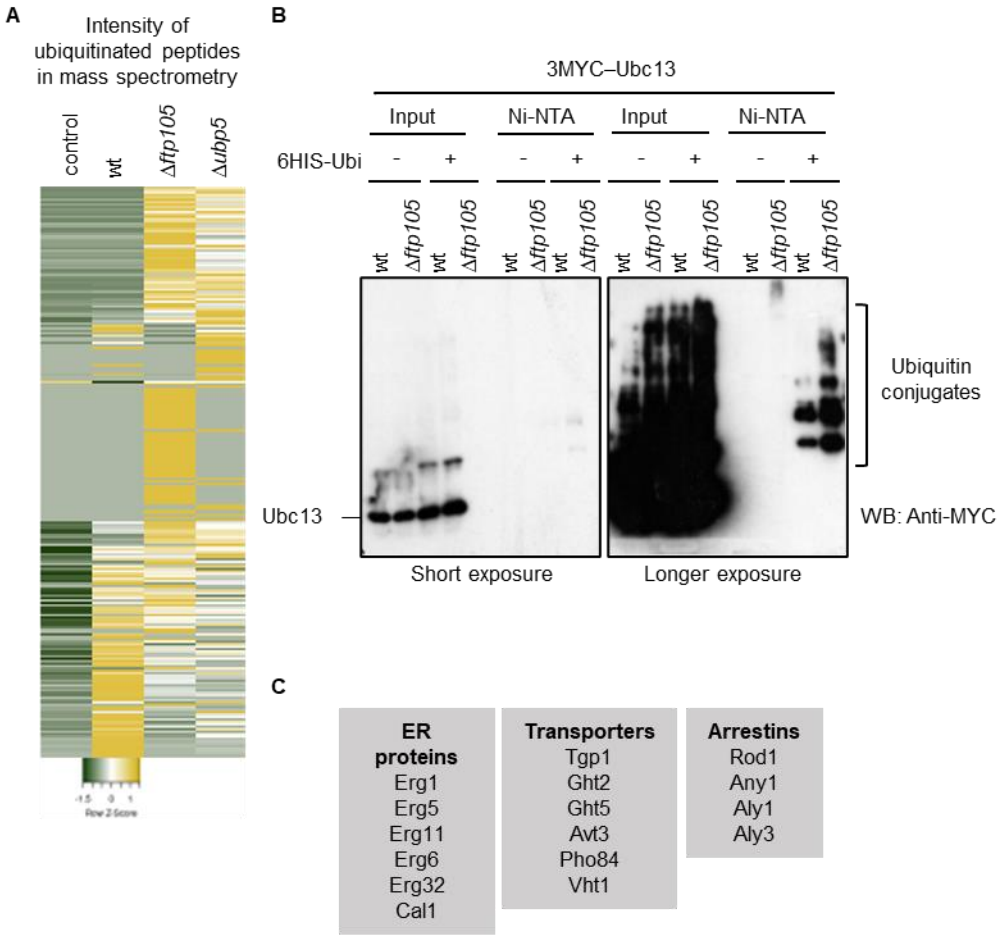


Figure 2.4.6: Ubiquitinated proteome of Ftp105 and Ubp5.

A) Analysis of total ubiquitinated proteins from wildtype, *Δftp105* and *Δubp5* strains using mass spectrometry. Heat-map shows intensity of the peptides obtained from the indicated strains. 6xHIS-tagged ubiquitin was expressed and Ni-NTA pulldown was done in denaturing condition. The control indicates wildtype strain with no 6xHIS-tagged ubiquitin construct transformed in it. Yellow colour represents accumulation, white denotes no change, and green shows reduction of intensities. B) Indicated expression clones were transformed in wildtype and *Δftp105* strains. Denaturing Ni-NTA pulldown was performed and ubiquitinated pool of Ubc13 was blotted using anti-MYC antibody. C) Diverse substrates of Ftp105 and Ubp5.

2.4.7 Ftp105-Ubp5 complex ensures localization of the glucose transporter Ght5 to plasma membrane

As shown previously, *ftp105* and *ubp5* deletion strains grew slower in low glucose conditions (Figure 2.4.5B). The slow growth could be due to the absence of a high affinity glucose transporter to transport the glucose into the cells. Ght5 is a key high affinity glucose transporter in *S. pombe*. From the eight glucose transporters, it is the only transporter essential for growth of cells in low glucose conditions (Saitoh et al. 2014). Ght5 is one of the substrates of the Ftp105-Ubp5 complex (Figure 2.4.6C). To study Ght5 localization in wildtype, *Δftp105* and *Δubp5* strains, Ght5 was endogenously tagged with a C-terminal GFP tag in these strains. Localization was studied in high and low glucose conditions (glucose amount was reduced from 111mM (2%) to 4.4mM (0.08%) for low glucose

conditions). In response to changes in glucose conditions, Ght5 is regulated with respect to its transcription and subcellular localization (Saitoh et al. 2014). We found that *ftp105* and *ubp5* deletion strains showed localization of Ght5 in the form of cytoplasmic punctate as opposed to the plasma membrane localization observed in wildtype strain (Figure 2.4.7A, B). Ght5 protein levels in different strains were checked by western blot analysis (Figure 2.4.7C). The appearance of more free GFP in the mutants, compared to the full length protein, indicates that Ght5 is degraded in the vacuole in the absence of Ftp105 and Ubp5. Thus, the Ftp105-Ubp5 complex ensures localization of Ght5 to plasma membrane and control its degradation in the vacuoles.

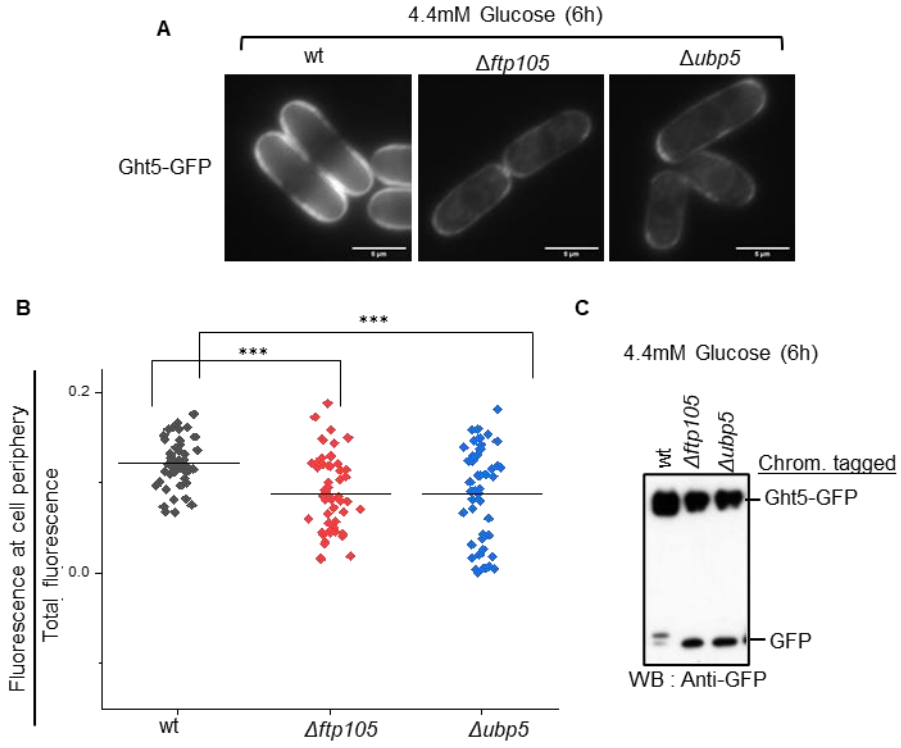


Figure 2.4.7: Ftp105-Ubp5 complex dependent localization of a glucose transporter, Ght5.

A) Subcellular localization of Ght5 in wildtype, *Δftp105*, and *Δubp5* strains. All the strains were tagged with Ght5-GFP and imaged in low glucose condition (4.4mM) for 6 hours. Scale bar = 5µm. B) From the experiment presented in A, quantification of the ratio of fluorescence at the cell periphery over total fluorescence at 6 hours in low glucose condition (4.4mM). n>50 (see Materials and Methods). C) Western blot analysis for the detection of Ght5 using anti-GFP antibody in conditions similar to Figure. 2.4.7A.

2.4.8 Ftp105-Ubp5 may control the trafficking of Ght5 from Golgi to Vacuole

Transporters are known to be regulated at multiple levels. When the nutrient is excess, the cognate transporter undergoes endocytosis and is either degraded in the vacuole or is part of the recycling endosome. It has also been shown that the newly synthesized transporters at the TGN can be rerouted directly to vacuole in conditions that trigger endocytosis of the transporter (Haguenauer-Tsapis

and André 2004b). To identify the route that Ght5 takes to vacuole in the absence of Ftp105 and Ubp5, we blocked endocytosis by deleting *vrp1* in the background of *ftp105* deletion.Vrp1 encodes the yeast WASP-interacting protein(WIP) homolog, verprolin and is essential for the process of endocytosis (Munn et al. 1995) (Figure 2.4.8A). The localization of Ght5 in the double mutant is similar to that of *ftp105* deletion (Figure 2.4.8B). This indicates that the cytoplasmic punctate localization of Ght5 is not due to increased endocytosis. The plausible route that Ght5 takes is the Golgi to vacuole route. We then checked whether the degradation of Ght5 is via the multivesicular body (MVB) pathway. ESCRT proteins (Endosomal Sorting Complex Required For Transport) are involved in the biogenesis of MVB (MacGurn, Hsu, and Emr 2012). We inhibited the formation of MVB by deleting *sst4* in the background of *ftp105* deletion. Sst4 is part of ESCRT-0 which is critical in the formation of MVB (Iwaki et al. 2007) (Figure 2.4.8C). The localization defect observed in *ftp105* deletion is rescued when the MVB formation is blocked (Figure 2.4.8D). However, more experiments are required to confirm whether the rescue in the localization of Ght5 also rescues the growth defect observed in low glucose in these mutants. This indicates that Ght5 is degraded via the MVB pathway. Thus, Ght5 is likely trafficked from Golgi to vacuole in the absence of Ftp105 and is degraded in the vacuole via the MVB pathway.

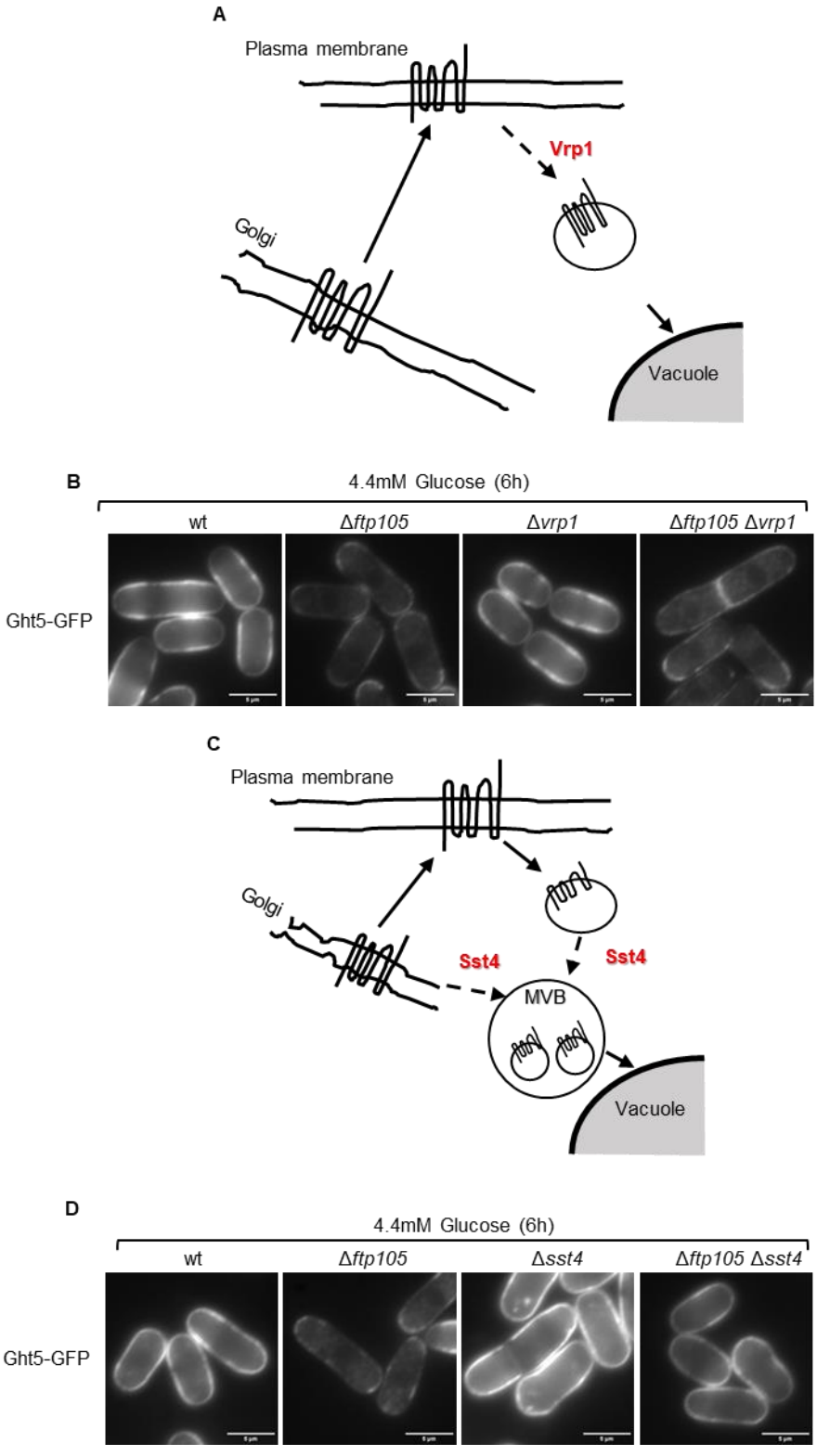
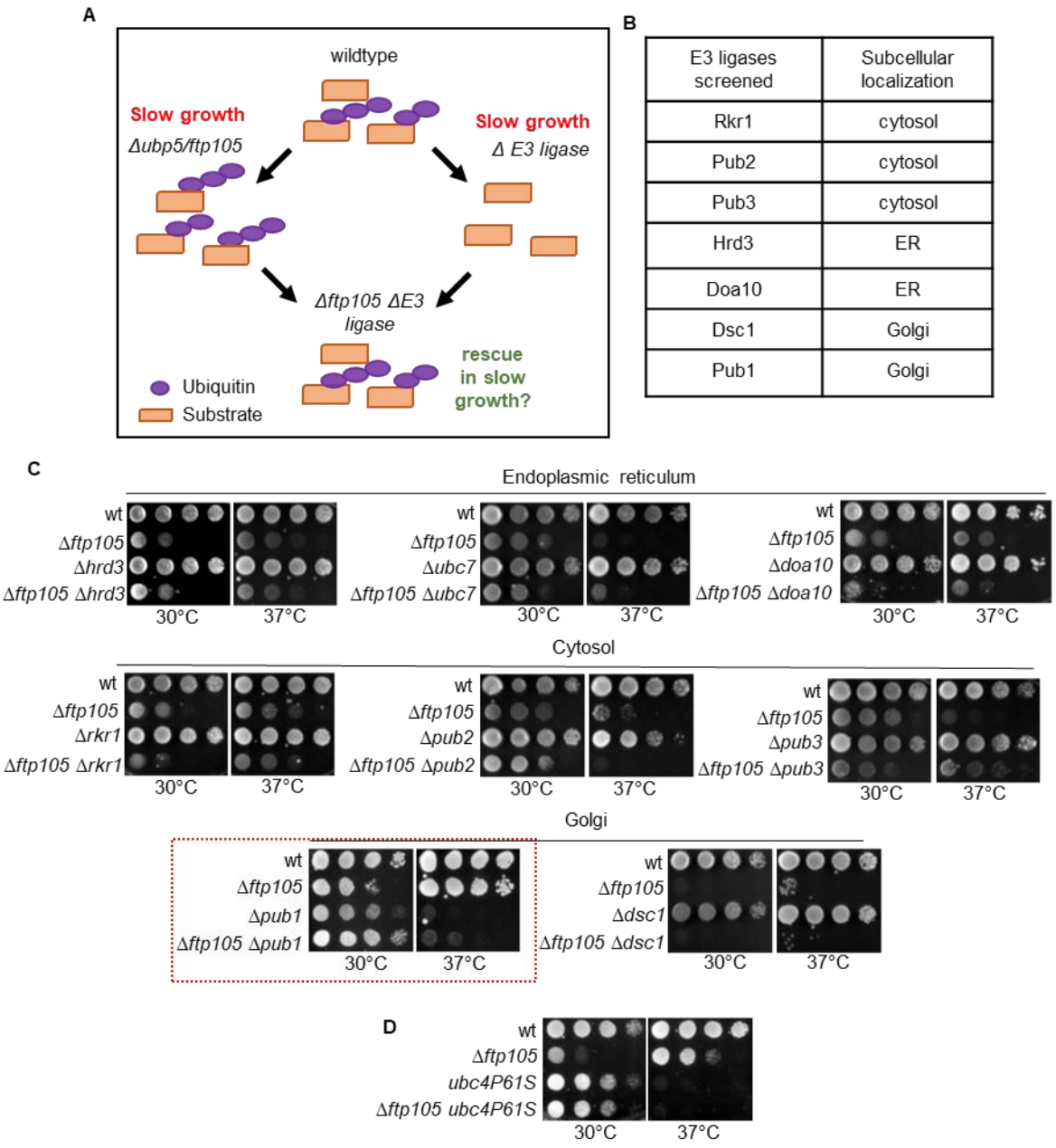


Figure 2.4.8: Ftp105-Ubp5 complex may control trafficking of Ght5 from Golgi to vacuole.

A) Schematic representation of the pathway which involves the function of Vrp1. B) Subcellular localization of Ght5. Ght5 is endogenously tagged with GFP in all indicated strains and imaging was done after treating the cells in low glucose (4.4mM) for 6 hours. Scale bar = 5µm. C) Schematic representation of the pathway which involves the function of Sst4. D) Assay is similar to B).

2.4.9 Identification of enzymes responsible for Ght5 ubiquitination

Ftp105 and Ubp5 was required for the plasma membrane localization of Ght5 by the process of deubiquitination. Then, the next question arose was what ubiquitinates Ght5. For this, we performed a targeted screen to identify the E3 ligase that ubiquitinates the substrates of Ftp105 and Ubp5. We chose selected E3 ligases localized in the endoplasmic reticulum, Golgi, and cytosol (Figure 2.4.9B). We also took Ubc7, a E2 conjugating enzyme of the ER-localized E3 ligase Hrd1, for the screen (Cohen et al. 2015). The idea was that DUBs' role on substrates would become redundant in the absence of the E3 ligase that ubiquitinates it. E3 ligases were co-deleted in the background of *ftp105* deletion and the double mutants were tested for growth rescue (Figure 2.4.9A). We performed the screen with *ftp105* deletion strain as both *ftp105* and *ubp5* deletion strains showed mislocalization of Ght5. We observed that the deletion of Pub1 rescued the growth defect in Ftp105 mutant (Figure 2.4.9C). Pub1 is known to regulate the endocytosis and localization of amino acid transporters in *S. pombe* (Y. Nakase et al. 2013a)(Aspuria and Tamanoi 2008; M. Nakase et al. 2012; Nakashima et al. 2014). The budding yeast homolog of Pub1, Rsp5 is the master regulator that regulates the fate of wide variety of transporters (Lauwers et al. 2010). Hxt6 and Hxt7, the *Saccharomyces cerevisiae* homolog of Ght proteins undergo endocytosis and degradation in high glucose conditions in a process mediated by Rsp5 (Krampe et al. 1998). So, Pub1 may be one of the E3 ligases which ubiquitinates the substrates of Ftp105 and Ubp5. The E2 conjugating enzyme of Pub1 is Ubc4 (Nuber and Scheffner 1999). The deletion of Ubc4 is lethal in *S. pombe.* So, we took a temperature-sensitive allele of *ubc4* (Hiroaki et al. 2003) and made the deletion of Ftp105 in this background. Interestingly, the *ts* allele of *ubc4* also recued the growth defect observed in the absence of Ftp105 (Figure 2.4.9D). This indicates that Pub1 and Ubc4 are one of the E3/E2 complex proteins involved in the ubiquitination substrates of Ftp105 and Ubp5.



E3 ligases screened	Subcellular localization
Rkr1	cytosol
Pub2	cytosol
Pub3	cytosol
Hrd3	ER
Doa10	ER
Dsc1	Golgi
Pub1	Golgi

Figure 2.4.9: Screen for the putative E3 ligase.

A) Schematic describing the functioning of the screen. B) List of the E3 ligases screened. C) Double deletions were made with the indicated E3 ligases and growth assay was performed in synthetic media in different temperature conditions Red dotted box indicates the growth assay of *Δftp105 Δpub1* where we observed a growth rescue. D) Growth assay is similar to (B).

2.4.10 Pub1 promotes sorting of Ght5 to Vacuole

We then questioned whether the localization defect of Ght5 is also rescued in the absence of Pub1. For this, Ght5 was endogenously tagged with a C-terminal GFP tag in *Δpub1* and *Δftp105 Δpub1* and its localization was checked in low glucose conditions (similar to Figure 2.4.7A). We observed plasma membrane localization of Ght5 in *Δftp105 Δpub1* strain as opposed to the cytoplasmic

localization observed in *Δftp105* strain (Figure 2.4.10A). In the absence of Pub1, Ght5 protein level was restored in the *ftp105* deletion strains (Figure 2.4.10B). Thus, Pub1/Ubc4 ubiquitinates Ght5 which is then deubiquitinated by Ftp105 and Ubp5 leading to its localization to plasma membrane.

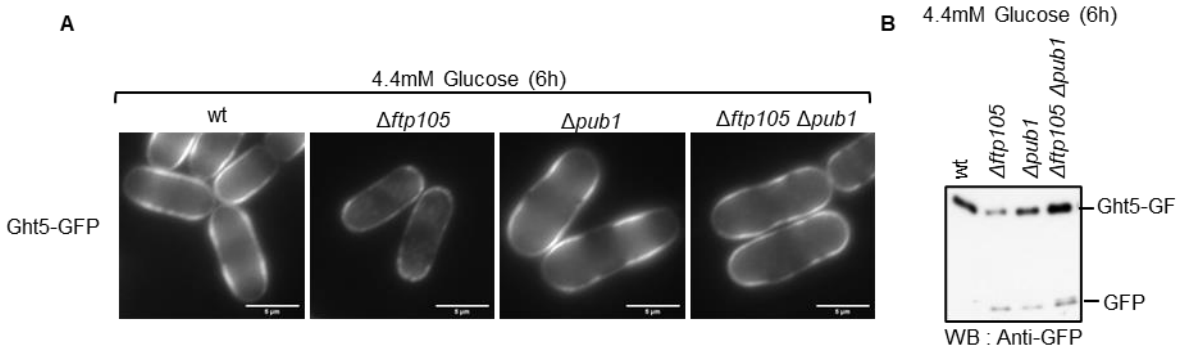


Figure 2.4.10: Pub1 is one of the putative E3 ligases that ubiquitinates Ght5.

A) Subcellular localization of Ght5. Ght5 is endogenously tagged with GFP in all indicated strains and imaging was done after treating the cells in low glucose (4.4mM) for 6 hours. Scale bar = 5µm. B) Western blot analysis for the detection of Ght5 using anti-GFP antibody in conditions similar to (A).

2.4.11 Rod1 and Aly3 are the arrestin-related trafficking adaptors involved in the sorting of Ght5 to vacuole

Rsp5 is known to regulate the fate of transporters by binding to a group of adaptor proteins of the ART family (Arrestin-Related Trafficking adaptors). The ART family proteins or arrestins confer Rsp5 the ability to ubiquitinate transporters in response to different nutrient conditions (Barata-Antunes et al. 2021). In *S. pombe,* Ght5 is known to be ubiquitinated and endocytosed in nitrogen poor conditions in a process dependent on the arrestin, Aly3 (Toyoda et al. 2021). To screen for the ARTs involved in the trafficking of Ght5 from Golgi to vacuole, we made double mutants with four ARTs, Aly1, Aly3, Rod1 and Any1 respectively in the background of *ftp105* deletion strain. Rod1 and Aly3 have been shown to regulate the trafficking of glucose transporters in both budding yeast and fission yeast (Becuwe et al. 2012; Hovsepian et al. 2017; Llopis-Torregrosa et al. 2016; Toyoda et al. 2021). Any1 has been shown to be localized at the Golgi (Y. Nakase et al. 2013b). Also in budding yeast, the homolog of Aly1, Aly2 has been shown to regulate the trafficking of amino-acid transporter to the Golgi (O'Donnell et al. 2010). So, we started our screen with these four arrestins. We endogenously tagged Ght5 with a C-terminal GFP tag in all the respective arrestin and *ftp105* double mutant strains. We then performed microscopy to check the localization of Ght5 in low glucose condition similar to as performed in Figure 2.4.7A. The localization defect observed in *Δftp105* strain is rescued in the absence of Aly3 and Rod1 (Figure 2.4.11A). We performed western blot analysis and found that the Ght5 protein level is also restored in the *ftp105* deletion strains in the absence of Rod1 and Aly3 (Figure 2.4.11B). Thus, Rod1 and Aly3 in concert with Pub1/Ubc4 may ubiquitinate Ght5 for trafficking it to the vacuole

in the absence of Ftp105. The Ftp105-Ubp5 complex might counteract the activity of Rsp5/Pub1 and ARTs, Aly3 and Rod1 in regulating Ght5 localization (Figure 2.4.11C).

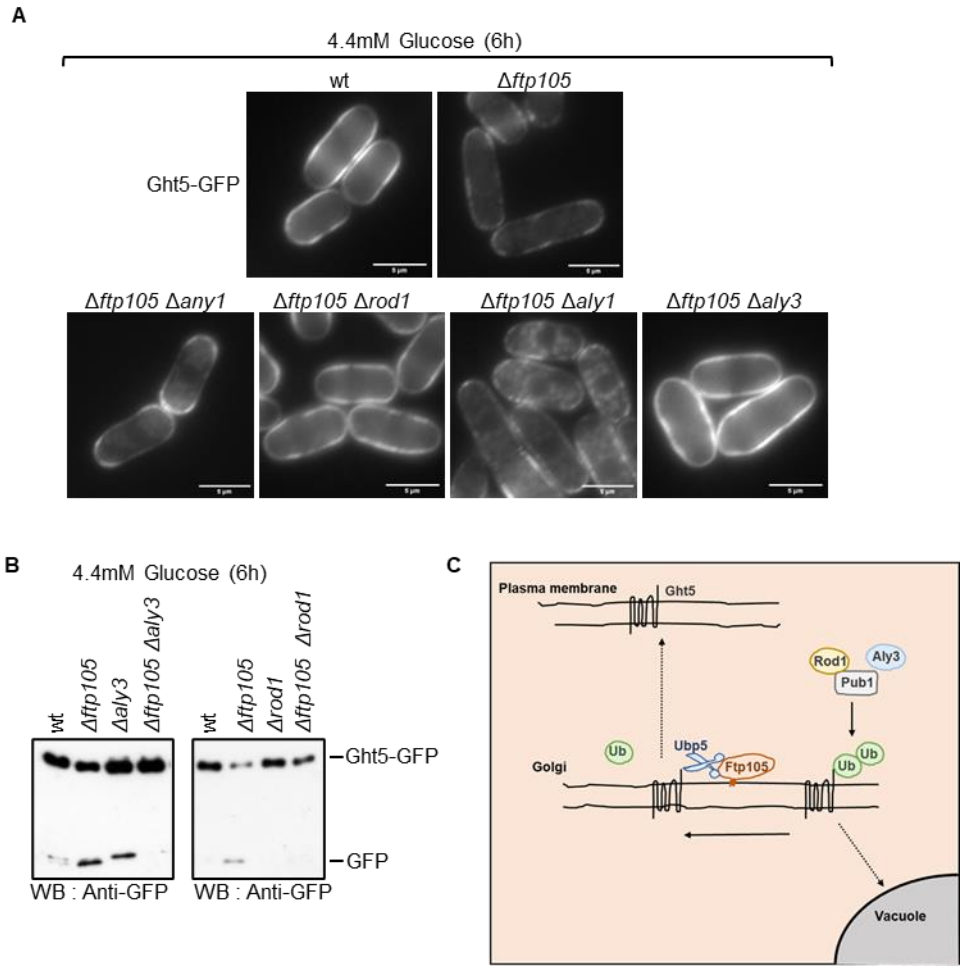


Figure 2.4.11 Rod1 and Aly3 are the arrestin-related trafficking adaptors that sort Ght5 to the vacuole in the absence of Ftp105.

A) Subcellular localization of Ght5. Ght5 is endogenously tagged with GFP in all indicated strains and imaging was done after treating the cells in low glucose (4.4mM) for 6 hours. Scale bar = 5µm. B) Western blot analysis for the detection of Ght5 using anti-GFP antibody in conditions similar to Figure. 11A. C) Proposed model of Ftp105-Ubp5 dependent deubiquitination of Ght5.

2.5 DISCUSSION

2.5.1 A novel DUB module at the Golgi

In our study, we identified the role of a novel DUB module at the Golgi. Using microscopic, genetic and biochemical studies, we found that the deubiquitinating enzyme, Ubp5, and its interacting partner Ftp105 regulate the trafficking of glucose transporter to the plasma membrane. Ubp5 localizes to Golgi through its interaction with Ftp105. Ftp105 is anchored to the Golgi membrane via N-terminal myristoylation modification.

The Golgi apparatus, particularly the trans-Golgi network (TGN), acts as a compartment where neosynthesized cargoes are sorted to different compartments and also where cargo recycling happens (Kienzle and von Blume 2014). Although the role of several arrestin-related trafficking adaptors and E3 ligases are well-characterized, the role of deubiquitinating enzymes which reverses the ubiquitin mark is not known. Ubp5 can serve as the DUB which regulates the cargo trafficking at TGN. Thus, Ubp5 may regulate the fate of all cargoes sorted from the TGN.

DUB's activity is known to be regulated by its localization and post-translational modifications (Sahtoe and Sixma 2015). We have found that Ftp105 affects the localization of Ubp5. It will be interesting to see if Ftp105 affects its deubiquitinating activity also. Previous report has suggested that the loss of Ftp105 does not affect the activity of Ubp5 towards the artificial substrate Ub-AMC (Kouranti et al. 2010). However, further studies are required to delineate the role of Ftp105 on Ubp5.

The ubiquitination system has to be regulated in response to changes in nutritional status of the cell. The pathways regulating the ubiquitin conjugation machinery in different conditions is well characterized (Barata-Antunes et al. 2021). In budding yeast, TORC1 activates Art1,an arrestin-related trafficking adaptor, to promote the ubiquitination-mediated endocytosis of Can1, an amino acid transporter in nutrient-rich condition (MacGurn et al. 2011). The mechanism by which the activity of the Ftp105-Ubp5 complex is regulated in response to environmental cues will help us in better understanding how the transporters' fate is regulated.

The localization of DUBs is known to be modulated by post-translational modifications, especially via phosphorylation (Herhaus et al. 2015; Mueller et al. 2009; Yuan et al. 2010). A study investigating the phosphorylation status of DUBs in fission yeast revealed that Ubp5 and Ftp105 are phosphorylated in multiple sites (McLean, Kouranti, and Gould 2011). The phosphorylation status may dictate the localization of Ftp105 and Ubp5 depending on the environmental cues which can affect its activity. Further studies are needed to ascertain the role of phosphorylation on this DUB module.

2.5.2 Regulation of a glucose transporter

Mass spectrometric analysis revealed the ubiquitinated substrates enriched in the absence of Ftp105 and Ubp5. One of the substrates identified was the high affinity glucose transporter, Ght5. Loss of Ftp105 and Ubp5 leads to increased degradation and mis-sorting of Ght5 into the vacuole. Thus, in glucose limiting conditions, Ftp105 and Ubp5 are required for the proper sorting of Ght5 to the plasma membrane.

Many newly synthesized transporters are stored in Golgi or other components of endosomal pathway before being sorted into the plasma membrane. One of the well-studied examples is the sorting of high affinity glucose transporter, GLUT4 in humans. GLUT4 is stored in specialized storage vesicles called GLUT4 storage vesicles (GSV). Insulin triggers the exocytosis of GLUT4 to the plasma membrane (Leto and Saltiel 2012). The regulated secretion of GLUT4 shares many similarities to the DUB-dependent sorting of Ght5. Ubiquitination of Ght5 may act as the signal for storage at Golgi and deubiquitination in response to low glucose may trigger its exocytosis. Impaired sorting of GLUT4 leads to the development of type 2 diabetes (Saltiel and Kahn 2001). In mice, loss of HID-I, the homolog of Ftp105 shows diabetes-like symptoms as insulin secretion is affected (Du et al. 2016). It is tempting to postulate that HID-1 may also regulate the trafficking of GLUT4. As Ftp105 and Ubp5 are highly conserved across different species, understanding the mechanism of the functioning this DUB module may help in understanding and treating diabetes like metabolic syndromes.

2.5.3 Role of the E3 ligase and arrestin-related trafficking adaptors (ART)

Pub1 acts as one of the E3 ligases that ubiquitinates Ght5 and sort it into the vacuole. The degradation of Ght5 is not completely abolished in the absence of Pub1.This indicates that there are additional E3 ligases that ubiquitinate and degrade Ght5. In *S. pombe*, Pub1 has two more paralogs, Pub2 and Pub3 and Pub2 has been shown to have partially overlapping function with Pub1 (Tamai and Shimoda 2002). So, these E3 ligases might have redundant activity on Ght5.

Pub1 localizes to the Golgi as well as the plasma membrane in *S. pombe* (Y. Nakase et al. 2013b). The presence of both the E3 ligase and the DUB at the Golgi provides a platform to fine-tune the ubiquitination of transporters in response to change in environmental cues. DUB-E3 ligase interactions are well-characterized and they regulate a wide variety of signaling processes (Nielsen and MacGurn 2020). Pub1 and Ubp5 might represent a new DUB-E3 complex regulating the fate of transporters synthesized from Golgi.

Rod1 and Aly3 are the arrestin-related trafficking adaptors that regulate the sorting of Ght5 to vacuole. In *S. pombe,* Aly3 has been shown to promote the translocation of Ght5 from plasma membrane to vacuole in nitrogen poor conditions (Toyoda et al. 2021). In our study, we found that Aly3 along

with Rod1 regulates the trafficking of Ght5 from Golgi to vacuole in glucose-limiting conditions. This suggests that the arrestins may localize to Golgi to promote the ubiquitination of Ght5. In budding yeast, Rod1 localizes to the Golgi to sort Jen1, a monocarboxylate transporter to the vacuole in high glucose conditions (Becuwe and Léon 2014). So, Rod1 and Aly3 also might localize to the Golgi to ubiquitinate Ght5. In budding yeast, Rod1 triggers the endocytosis of hexose transporters in response to change in glucose concentrations. In low glucose conditions, Snf1, the yeast homolog of AMPK. phosphorylates Rod1 and inactivates it (Becuwe et al. 2012; Llopis-Torregrosa et al. 2016). In humans, the α arrestin TXNIP is phosphorylated by AMPK which leads to its degradation and prevents its interaction with the GLUT1 (Ning Wu et al. 2013). It will be interesting to see whether Rod1 and Aly3 are also phosphorylated by *S. pombe* AMPK Ssp2 and what is their phosphorylation status in the absence of Ftp105 and Ubp5.

In our mass spectrometric analysis, we found that the arrestins, Aly3 and Rod1 are also hyperubiquitinated. Rsp5-mediated hyperubiquitination of ARTs are required for its activity (Léon and Haguenauer-Tsapis 2009). Ftp105-Ubp5 complex may deubiquitinate the ARTs also to further attenuate the Golgi to vacuole sorting of transporters.

2.5.4 Possible mechanism of the DUB module

The localization of Ght5 in low glucose condition is affected in the loss of Ftp105 and Ubp5. Low glucose condition stimulates the transcription and plasma membrane localization of Ght5 in *S. pombe* (Saitoh et al. 2014). In high glucose condition, the activity of Pub1 and the arrestin-related trafficking adaptors, Rod1 and Aly3, might sort Ght5 into the vacuole. And the activity of Ftp105-Ubp5 might be kept inactive in the high glucose condition by an unknown mechanism. Ubiquitination has been shown as a signal for storing transporters at the Golgi (Y. Nakase et al. 2013a). So, in high glucose conditions where there is little requirement of glucose transporters, Ght5 also might be stored at the Golgi via the ubiquitination by Pub1. When the glucose concentration changes, the DUB module becomes active and deubiquitinates Ght5 for its accurate sorting to the plasma membrane. Thus, the DUB mediated regulation of Ght5 might provide a faster response to combat glucose-related stresses (Figure 2.5.4).

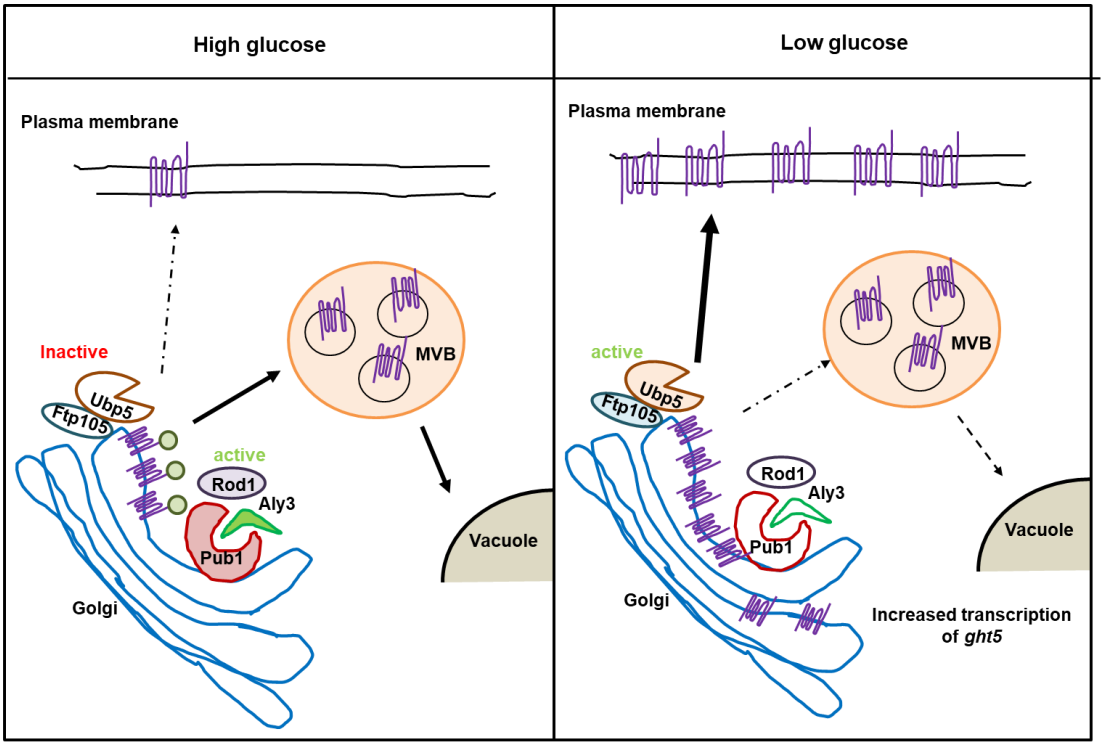


Figure 2.5.4: Proposed mechanism of the DUB module at Golgi.

We proposed that in high glucose condition, Ftp105-Ubp5 complex is inactive which leads to the ubiquitination of Ght5 by Pub1 and arrestin-related trafficking adaptors, Rod1 and Aly3. However, the activation of Ftp105-Ubp5 complex in low glucose leads to proper localization of Ght5 in the plasma membrane.

2.5.5 Additional targets of the DUB module

In our analysis, we found more plasma membrane transporters as substrates of the DUB module. One such substrate is Pho84, a high affinity phosphate transporter (Bun-Ya et al. 1991). Initial analysis from our lab revealed that Pho84 is also degraded in the vacuole in absence of Ftp105 and Ubp5. The Ftp105-Ubp5 mediated regulation of transporters involved in transporting two different metabolites strongly suggests that the DUB module can act as gate-keepers which prevents the mis-sorting of different cargoes arising from the Golgi apparatus.

We also observed that the proteins involved in the biosynthesis of ergosterol as targets of the DUB module. These proteins are ER membrane proteins that synthesize ergosterol. Ergosterol levels are known to regulate the endocytic and exocytic activity (Degreif et al. 2019). The measurement of the level of ergosterol in the absence of Ftp105 and Ubp5 will give us insights into the role of these proteins in the regulation of ergosterol biosynthesis.

2.6 CONCLUSION

In this chapter of my thesis, I found that the Ftp105-Ubp5 module function at the Golgi to regulate the trafficking of glucose transporter, Ght5. Ftp105 localizes to the Golgi via N-terminal myristoylation. Ubp5 is epistatic over Ftp105 and Ftp105 mostly functions via its interaction with Ubp5. Using mass spectrometric analysis, we found that the substrates of Ftp105 and Ubp5. One of substrate is the high affinity glucose transporter, Ght5. Ght5 is mislocalized in the absence of Ftp105 and Ubp5 and is degraded in the vacuole. Pub1 along with the ARTs, Rod1 and Aly3, ubiquitinate Ght5 to sort it into the vacuole. Direct ubiquitination assays are necessary to further strengthen the finding.

3 MATERIALS AND METHOD

3.1 Materials

3.1.1 Chemicals and plastic wares

All chemicals used in the study were either of analytical or molecular biology grades and were obtained from commercial sources. Media components, fine chemicals, and reagents were purchased from Sigma Aldrich, USA, HiMedia, India, Merck. Ltd, USA, Difco, USA and Formedium, UK. All plastic wares used for molecular biological and bacteriological works were purchased from Abdos labtech, India, and Tarsons, India.

3.1.2 Molecular biology reagents

Enzymes (Restriction enzymes, T4 DNA ligase, Alkaline Phosphatase (CIP), Phusion DNA polymerase, Taq DNA polymerase, Vent DNA polymerase and other modifying enzymes), their buffers, dNTPs, DNA and protein molecular weight markers were purchased from New England Biolabs, Invitrogen, Sigma Aldrich and Thermo Fisher Scientific. Gel-extraction and plasmid miniprep kits were obtained from Favorgen and Bioneer. RNA isolation kits were procured from, Invitrogen, and Applied Biosystems.

3.1.3 Antibodies and beads

The antibodies anti-Myc (polyclonal), anti-FLAG M2 (Clone M2), anti-FLAG (polyclonal), anti-haemagglutinin (HA, clone HA-7), anti-HA (polyclonal), anti-rabbit-HRP and anti-mouse-HRP (raised in Goat) were obtained from Sigma Aldrich and anti-GFP (monoclonal) and anti-Ubiquitin (monoclonal) were obtained from Santa Cruz, USA. Antibody-coupled beads Anti-HA-rabbit (H6908), and anti-FLAG (A2220) were also from the same source. HisPur™ Ni-NTA Magnetic Beads (88831) was obtained from Thermo Fisher Scientific, and Ni-NTA Magnetic Agarose Beads (36113) was obtained from Qiagen.

3.1.4 Media

All the media, buffers and stock solutions were prepared using Millipore water and were sterilized, as recommended, either by autoclaving at 15 lb/inch^2 (psi) pressures at 121 ºC for 15 minutes or by using membrane filters (HiMedia, India) of pore size 0.2 μ (for heat-labile compounds).

For making agar plates, 1.5% agar (HiMedia, Formedium) was added.

Luria-Bertani (LB) medium: 25 g of Formedium LB (LMM0102) was added to 1 liter of de-ionised water and was autoclaved. Desired antibiotics ampicillin (100 µg/ml) or kanamycin (50 µg/ml) were added as per requirements.

Yeast-extract with supplements (YES) medium: Per liter, 5 g yeast extract, 2 g casamino acids, 30 g glucose, 0.1 g adenine, 0.1 g uridine, 0.1 g leucine, 0.1 g histidine, 15 g agar was added; when selecting for the Kan-MX4 marker using G418/geneticin resistance (G-418-25), G418 plates were made by dissolving to make it to final concentration of 200 mg/l. For Nat-NT2 cassette, Nat plates with 75 µg/ml (cat no N-500-1), and for Hph-NT1 cassette, hygromycin plates with 100 µg/ml (cat no H-270-1) were made. All the antibiotics were obtained from GOLDBIO, USA.

Synthetic defined (SD) media: Per liter, 6.7 g yeast nitrogen base, 2 g of required supplements dropout mixtures for auxotrophies (e.g., leucine, adenine, uracil), 20 g glucose, are added as required and for making plates, 15 g agar was used.

Edinburgh minimal medium (EMM) medium: Per liter, 12.3 g EMM broth without dextrose *(*Formedium PMD0410), 75 mg histidine, 75 mg adenine, 80 mg uracil, 225 mg leucine, and 20 g glucose were added, and for plates 15 g agar was used. Expression constructs under the *nmt81* promoter were induced in the EMM medium.

3.1.5 Buffers and stock solutions

- *Cell lysis buffer (yeast genomic DNA isolation)*: 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl (pH-8.0), and 1 mM EDTA.

- *10x Tris-EDTA (TE) buffer (pH 8.0)*: 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA.

- *50x TAE:* Per liter: 242 g Tris base, 57. 1 ml glacial acetic acid, and 100 ml of 500 mM EDTA (pH 8.0). Autoclaved and stored at room temperature.

- *High Urea (HU) buffer:* 8M urea, 5% SDS, 200 mm Tris pH 6·8, 1 mM EDTA, with bromophenol blue, and 1.5% dithiothreitol (DTT) was added before use.

- *30% Acrylamide Mixutre:* 29.2% acrylamide, and 0.8% methylbisacrylamide was obtained from Bio-Rad or HiMedia.

- *Resolving Gel (12%):* 1.25 ml 1.5 M Tris-HCl (pH 8.8), 50 µl 10% SDS, 2 ml 30% acrylamide solution, 50 µl 10% ammonium persulphate (APS), 5 µl Tetramethylethylenediamine (TEMED), and 1.7 ml water.

- *Stacking Gel (4%):* 0.5 ml 0.5 M Tris-HCl (pH 6.8), 20 µl 10% SDS, 0.26 ml 30% acrylamide solution, 40 µl 10% APS, 4 µl TEMED, and 1.2 ml water.

- *Gradient gel*: Two different gel mixes of 4% and 15% were prepared. For 15% mix: 1.145 ml H_2O, 1.25 ml 1.5M Tris-Cl (pH 8.8), 50 µl 10% SDS, 2.5 ml 30% acrylamide solution, 50 µl 10% ammonium persulphate (APS), 5 µl Tetramethylethylenediamine (TEMED). Both the mix were added to the gradient mixer and the gel was casted.

- *10x SDS buffer (pH 8.3):* Per liter, 30 g Tris base, 144 g glycine, and 10 g SDS.

- *20x MOPS buffer (pH 7.7):* Per liter, 50 mM MOPS, 50 mM Tris base, 0.1% SDS, and 1 mM EDTA.

- *10x Semi-dry transfer buffer:* Per liter, 29.3 g Glycine, 58.2 g Tris base, and 4 g SDS. For transfer, 1x buffer with 10% methanol was used.

- *10x Phosphate-Buffered Saline (PBS) buffer (pH 7.6):* Per liter, 80 g NaCl (1.37 M), 2 g KCl (27 mM), 17.7 g $Na_2HPO_4.2H_2O$ (100 mM), 2.45 g KH_2PO_4 (18 mM). For washing, 1x PBS with 0.1% tween 20 was used.

- *IP Cell-lysis buffer:* 150 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-Cl (pH-7.5), 5%-10% glycerol, 1% Triton X-100, 1 mM PMSF, and one protease inhibitors tablet per 50 ml buffer (88266 from Thermo Fisher Scientific, USA).

- *IP buffer backbone:* 150 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris-Cl (pH-7.5), 5%-10% glycerol.

- *IP Wash buffer 1:* IP Cell lysis buffer diluted 10 times with IP buffer backbone.

- *IP Wash buffer 2:* IP buffer backbone with 1% Triton X-100.

- *SORB (pH 8):* 100 mM lithium acetate, 10 mM Tris–HCl (pH 8), 1 mM EDTA (pH 8), 1M sorbitol, filter sterilized and stored at room temperature.

- *40% PEG:* 100 mM lithium acetate, 10 mM Tris–HCl (pH 8), 1 mM EDTA (pH 8), 40% PEG, filter sterilized and stored at 4°C.

- *Salmon sperm DNA (10 mg/ml):* It was denatured at 100°C for 10 minutes, cooled on ice and stored at -20°C. For yeast competent cell preparation, 40 µl of denatured salmon sperm DNA was used per 50 ml of culture.

3.1.6 *S. pombe* strains and plasmids

A complete list of strains and plasmids utilized in this study is given in Tables 3.1 and 3.2 and reporters used in this study is given in Table 3.3.

Table 3.1. Plasmids used in this study.

Plasmid number	Plasmid used in this study	Description	Ref.
D318	*pREP81x-3MYC–rap1 cDNA*	*S. pombe rap1* cDNA in pREP81x with 3MYC tag at the N-terminus	This study
D319	*pREP81x-3MYC–rap1 genomic*	*S. pombe rap1* genomic sequence in pREP81x with 3MYC tag at the N-terminus	This study

D322	*pREP81x-3MYC–rap1 genomic (stop codon removed)*	*S. pombe rap1* genomic sequence where 5' splice site of intron-2 is mutated to GTAAGT in pREP81x	This study
D321	*pREP81x-3MYC–psf3 genomic–3FLAG*	*S. pombe psf3* genomic sequence in pREP81x with 3MYC tag at the N-terminus and 3FLAG tag at the C-terminus	This study
D325	*pREP81x-3MYC–mcs2 genomic–3FLAG*	*S. pombe mcs2* genomic sequence in pREP81x with 3MYC tag at the N-terminus and 3FLAG tag at the C-terminus	This study
D406	*pREP81x-3MYC–hif2 genomic–3FLAG*	*S. pombe hif2* genomic sequence in pREP81x with 3MYC tag at the N-terminus and 3FLAG tag at the C-terminus	This study
D403	*pREP81x-3MYC–rxt2 genomic–3FLAG*	*S. pombe rxt2* genomic sequence in pREP81x with 3MYC tag at the N-terminus and 3FLAG tag at the C-terminus	This study
D1174	*pGADC1-Ftp105*	*S. pombe ftp105* in pGBDUC1 vector	This study
D1175	*pGBDUC1-Ubp5*	*S. pombe ubp5* cloned in pGADC1 vector	This study
D1091	*pFtp105 promoter Ftp105-3XFLAG*	*S. pombe ftp105* under its own promoter(1000bp) with 3FLAG tag at the C-terminus	This study
D1092	*pFtp105 promoter Ftp105(G2A)-3XFLAG*	D1091 with a mutation of G2A	This study
D1093	*pFtp105 promoter Ftp105-GFP*	*S. pombe ftp105* under its own promoter(1000bp) with GFP tag at the C-terminus	This study
D1094	*pFtp105 promoter Ftp105(G2A)-GFP*	D1094 with a mutation of G2A	This study

D1066	*pDual-eno1-6XHIS-ubi*	*S. pombe Ubiquitin* with 6HIS tag at the N-terminus	Li-Lin'lab
D1147	*pUbc13 promoter 3XMYC-Ubc13*	*S. pombe ubc13* under its own promoter(1000bp) with 3FLAG tag at the C-terminus	This study

Table 3.2. *S. pombe* strains used in this study.

Strain	Genotype	Ref.
JY741	h– *ade6-M216 leu1 ura4-D18*	
JY746	h– *ade6-M216 leu1 ura4-D18*	
SP20	h+ *Δsde2::natnt2*	(Thakran et al. 2018)
SP52	h+ *Δubp15::kanMX4, Δubp5::natNT2*	(Thakran et al. 2018)
SP77	h– *sde2::sde2(AAK)-6HA:natNT2*	(Thakran et al. 2018)
SP82	h+ *sde2::sde2(K85M)-6HA:natNT2*	(Thakran et al. 2018)
SP140	h– *rap1-6HA:natNT2*	This study
SP143	h– *hif2-6HA:natNT2*	This study
SP145	h– *dsh1-6HA:natNT2*	This study
SP146	h+ *Δsde2::kanMX4, rap1-6HA:natNT2*	This study
SP149	h+ *Δsde2::kanMX4, hif2-6HA:natNT2*	This study
SP151	h+ *Δsde2::kanMX4, dsh1-6HA:natNT2*	This study
SP152	h+ *Δcay1::kanMX4, rap1-6HA:natNT2*	This study
SP155	h+ *Δcay1::kanMX4, hif2-6HA:natNT2*	This study
SP157	h+ *Δcay1::kanMX4, dsh1-6HA:natNT2*	This study
SP158	h+ *Δtls1::kanMX4, rap1-6HA:natNT2*	This study
SP161	h+ *Δtls1::kanMX4, hif2-6HA:natNT2*	This study
SP163	h+ *Δtls1::kanMX4, dsh1-6HA:natNT2*	This study

SP188	h+ *sde2::ubi–sde2-C-6HA:natNT2*	This study
SP285	h- Δ*ftp105::natNT2*	This study
SP55	*h+* Δ*ubp5::kanMX4*	Bioneer deletion library
SP515	h+ *ubp5–GFP:kanMX4*	This study
SP517	h- Δ*ftp105::natNT2, ubp5–GFP:kanMX4*	This study
SP516	h+ *ubp5–GFP-kanMX4, vrg4–mCherry:hphNT1*	This study
SP414	Δ*ftp105::natNT2,* Δ*ubp5::kanMX4*	This study
SP421	Δ*ftp105::natNT2,* Δ*ubp15::kanMX4*	This study
SP423	*h+*Δ*ubp5::kanMX4, pENO 6XHis-Ubiquitin:Leu*	This study
SP424	*h-*Δ*ftp105::natNT2, pENO 6XHis-Ubiquitin:Leu*	This study
SP425	*h-pENO 6XHis-Ubiquitin:Leu*	This study
SP567	*ght5-GFP:hphNT1*	This study
SP568	Δ*ftp105::natNT2, ght5-GFP:hphNT1*	This study
SP569	Δ*ubp5::kanMX4, ght5-GFP:hphNT1*	This study
SP475	Δ*vrp4::kanMX4, ght5-GFP:hphNT1*	This study
SP477	Δ*vrp4::kanMX4,* Δ*ftp105::natNT2, ght5-GFP:hphNT1*	This study
SP479	Δ*sst4::kanMX4, ght5-GFP:hphNT1*	This study
SP489	Δ*sst4::kanMX4,* Δ*ftp105::natNT2, ght5-GFP:hphNT1*	This study
SP446	Δ*pub1::kanMX4, ght5-GFP:hphNT1*	This study
SP448	Δ*pub1::kanMX4,* Δ*ftp105::natNT2, ght5-GFP:hphNT1*	This study
SP476	Δ*rod1::kanMX4,* Δ*ftp105::natNT2, ght5-GFP:hphNT1*	This study
SP488	Δ*aly3::kanMX4,* Δ*ftp105::natNT2, ght5-GFP:hphNT1*	This study
SP451	Δ*aly1::kanMX4,* Δ*ftp105::natNT2, ght5-GFP:hphNT1*	This study
SP450	Δ*any1::kanMX4,* Δ*ftp105::natNT2, ght5-GFP:hphNT1*	This study
SP532	Δ*hrd3::kanMX4,* Δ*ftp105::natNT2*	This study

SP533	Δ*rkr1::kanMX4, Δftp105::natNT2*	This study
SP534	Δ*doa10::kanMX4, Δftp105::natNT2*	This study
SP535	Δ*pub2::kanMX4, Δftp105::natNT2*	This study
SP536	Δ*pub3::kanMX4, Δftp105::natNT2*	This study
SP537	Δ*dsc1::kanMX4 Δftp105::natNT2*	This study
SP452	Δ*pub1::kanMX4, Δftp105::natNT2*	This study
SP323	h- *leu1-32 ura4-D18 ade6-M216 his3-D1 ubc4-P61S::ura4+*	(Seino et al. 2003)
SP346	h-*leu1-32 ura4-D18 ade6-M216 his3-D1 ubc4-P61S::ura4+ Δftp105::natNT2*	This study
SP56	*h+ Δubp15::kanMX4*	Bioneer deletion library
	h+ Δpub1::kanMX4	Bioneer deletion library
	h+ Δsst4::kanMX4	Bioneer deletion library
	h+ Δvrp1::kanMX4	Bioneer deletion library
	h+ Δrkr1::kanMX4	Bioneer deletion library
	h+ Δpub2::kanMX4	Bioneer deletion library
	h+ Δpub3::kanMX4	Bioneer deletion library
	h+ Δhrd3::kanMX4	Bioneer deletion library
	h+ Δubc7::kanMX4	Bioneer deletion library
	h+ Δdoa10::kanMX4	Bioneer deletion library
	h+ Δdsc1::kanMX4	Bioneer deletion library
	h+ Δany1::kanMX4	Bioneer deletion library
	h+ Δrod1::kanMX4	Bioneer deletion library
	h+ Δaly1::kanMX4	Bioneer deletion library
	h+ Δaly3::kanMX4	Bioneer deletion library

Table 3.3. Reporters used in this study.

Plasmid number	Splicing Reporter	Introns inserted in *ura4* gene (BP is shown in bold case letters, mutations are underlined)	Nucleotide(nt) between BP and 3'ss
D506	*peno-3MYC–ura4*	No intron	
D507	*rap1-i1*	GTATGGATTAATCATTATCAAAAAATTT**CTAAT**CATTATTATTTAG	14 nt
D508	*rap1-i2*	GTATGATCTTGCTTACCATTAATTGTTTTTTATTTTTTTT**CTAAC**ATTTTCCGCTTTCTATATTGGCGGCTACGGTTTCCTAG	39 nt
D510	*tho5-i1*	GTACGTGAAAGTTCCTTACCTTTTTTTTTTTTTTTTCAGATTTCAAAAGTCTTTGTTCATTTCTTT**CTAAC**CATTTTAATAG	12 nt
D511	*rap1-i2* (BP-3'ss dist. 12 nt)	GTATGATCTTGCTTACCATTAATTGTTTTTTATTTTTTTCTTCATTTTCCGCTTTCTATATTGTT**CTAAC**CGGTTTCCTAG	12 nt
D1067	*pyp3-i1* (BP-3'ss dist. 23 nt)	GTTAGGAATAAAAAGAAATTGCGGGATTGTAAACGGCTTTGGCCTTTTTTCTTTTCATCATTTTTTGTAACATAATTA**CTAAC**TTAGTCTTCTTTATTTATATAG	23 nt
D1068	*ftp105-i3* (BP-3'ss dist. 24 nt)	GTATGAAATTTTTTTTATTAACTTGTGTAAGACGCTTTTA**CTAAT**GTATTCGTTTTTATGTTTATTAG	24 nt
D1069	*ftp105-i3* (BP-3'ss dist. 12 nt)	GTATGAAATTTTTTTTATTAACTTGTGTAAGACGCTTTTACTATTGTATTCG**CTAAT**ATGTTTATTAG	12 nt
D666	*tho5-i1* (BP-3'ss dist. 21 nt)	GTACGTGAAAGTTCCTTACCTTTTTTTTTTTTTTTTCAGATTTCAAAAGTCTTTGTT**CTAAC**CTTTCTTTCCATTTTAATAG	21 nt

D1072	*tho5-i1* (competing 3'ss)	GTACGTGAAAGTTCCTTACCTTTTTTTTTTTTTTTT CAGATTTCAAAAGTCTTTGTT**C<u>TAAC</u>**CTTTCT<u>TTTA G</u>TTTTAATAG	12nt, 21nt

Table 3.4. Primers used for RT-PCR assays.

Primer Name	Primer sequence (5'-3')
rap1 Ex1 F	CCAAAAGCGATGGCTCGTCC
rap1 Ex3 R	AACCGAAGCAGACTTGGAAATC
act1 F	CCCCTAGAGCTGTATTCCC
act1 R	CCAGTGGTACGACCAGAGG
ura R	CTGTGTAGGAACCAGTAGCC
ura F	GTACAAAATTGCTTCTTGG
tho5 Ex1 F	CGATAACGCCCGTATACGGC
tho5 Ex2 R	CTGCTTTAAGGTGGAAAGTCG
psf3 Ex3 F	GTATCTATTCGGGACATAACCACAC
psf3 Ex5 R	CTACGAAGTGGAATTTTGCC
hif2 Ex1 F	CTGGAGATATTTGAAAGAATGC
hif2 Ex2 R	AGCTTTCGTCAGTGTCG
dsh1 Ex1 F	GGCAGAAAATAAGAAATTTTC
dsh1 Ex5 R	TTTTACACTGTCGCATCG
ftp105 Ex1 F	CGCGGGATCCATGGGAGGCCAAGAGTCAAA
ftp105 Ex4 R	GAGATGTCAATACAAGCAGC

3.2 Methods

3.2.1 *S. pombe* strain maintenance

Strains were cultured to stationary phase (around 1.5 OD_{600}) in YEL media at 30°C for 16–18 hours with shaking at 250 rpm, then mixed 1:3 with 50% (v/v) sterile glycerol and immediately stored at -80°C. For experiments, strains were revived from glycerol stocks on YES plates and maintained at standard growth conditions. The transformed yeast strains were selected and maintained on SD medium with supplements as per the requirement to keep the selective pressure on the plasmid. Strains used in this study are listed in Table 3.2.

3.2.2 Yeast genomic DNA isolation

Protocol was adapted from (Murray, Watson, and Carr 2016). Strains were grown to saturation phase till 1.5 OD_{600} in 5 ml YEL media at 30°C for 16–18 hours at 250 rpm. Cells were harvested by centrifugation at 3000 rpm for 5 min at room temperature and washed with distilled water. Cells were lysed by glass beads method in presence of 200 µl lysis buffer and 200 µl phenol:chloroform:isoamyl alcohol (PCI) by vigorous vortexing for 5 min with incubation of 1 min on ice. After vortexing, cells were suspended in 200 µl TE buffer and centrifuged at maximum speed for 10 min at room temperature. The supernatant was transferred to the new microcentrifuge tube with an equal volume of chloroform; vortexed and centrifugation was done for 5 min. The aqueous layer was again transferred to a fresh tube with 1 ml of 100% ethanol. The tube was gently mixed; incubated on ice for 10 min and centrifuged. After discarding the supernatant, the pellet was dissolved in 400 µl TE buffer with 6 µl of RNase A (5 mg/ml) and the tube was incubated at 37°C for 30 min. Then, 10 µl 4 M ammonium acetate with 1 ml 100% ethanol was added to the tube and incubated on ice for 10 min. Following precipitation, centrifugation was performed at maximum speed for 10 min. The pellet was washed with 1 ml 70% ethanol; dried in vacuum concentrator; resuspended in 50 µl TE buffer and stored at -20°C.

3.2.3 Preparation of *S. pombe* competent cells

Competent cells preparation was done following published protocols (Knop et al. 1999). Briefly, *S. pombe* cultures were grown in YEL media at 30°C with shaking for 16-24 hours and then reinoculated in fresh YEL media to an OD_{600} around 0.1–0.2. Cells were incubated at 30°C with shaking at 250 rpm till the OD_{600} nearly 0.5–0.7. The cells were harvested by centrifugation at 3000

rpm, for 5 min, room temperature then washed with sterile water and once with SORB. After centrifugation, SORB was removed and the pellet was resuspended in 360 µl SORB and 40 µl denatured salmon sperm DNA (10 mg/ml stock solution) per 50ml culture. Aliquots were made and competent cells were stored at -80°C.

3.2.4 Transformation of *S. pombe*

The transformation of *S. pombe* strains was carried out by lithium acetate method (Knop et al. 1999). 10 µl of competent cells were mixed with 1 µl-2 µl of plasmids in a sterile microcentrifuge tube and six-fold sterile 40% PEG was added. After vortexing, cells were incubated at 30°C for 30 minutes. Heat shock was given at 42°C for five minutes and cells were kept on ice for 5 minutes. After addition of 100 µl sterile water, whole solution was plated on selection plates and kept at 30°C for 3-4 days.

3.2.5 Complementation assays

To complement growth defect phenotypes of different mutant strains, competent cells were transformed with respective plasmids and plated on selection plates as described in (Thakran et al. 2018). The plates were incubated at 30°C till the growth of transformants was observed. After growth, the transformants were resuspended in sterile water and OD_{600} was measured. The transformants were diluted at five-fold serial dilution in a microtiter plate and dilution spotting was done on different plates as indicated in the figures and with 5 µg/ml thiamine and without thiamine for constructs with *nmt* promoter. Following spotting, the plates were kept at different conditions such as temperature until growth was observed.

3.2.6 Site-directed mutagenesis

All point mutations, insertions or deletions on plasmids were created by using specific-primers and high-fidelity KOD hotstart DNA polymerase (Sigma 71086). Both primers harbor the desired mutations flanked by unmodified nucleotide sequences and anneal to the same sequence on opposite strands of the plasmid. The mutagenesis PCR was done for 25 cycles using 10-50 ng of template. Then, the PCR mixture was treated with 1 µl Dpn1 at 37°C for 3 hours to digest the parent template. Subsequently, 10 µl of amplified product was transformed in 100 µl of XL1-Blue competent cells and plated on the selective antibiotic plate.

3.2.7 Overlap extension (SOE) PCR

All the insertions and deletions in *ura4* reporters and other plasmids discussed in this study were generated by overlap extension (SOE) PCR method (Bryksin and Matsumura 2010). Two fragments which we wanted to fuse were amplified by high-fidelity polymerases and they had overlapping regions. Then, using PCR products as template we performed SOE PCR with Vent polymerase using flanking primers.

3.2.8 Protein isolation by trichloroacetic acid (TCA) precipitation

Cells were grown to log phase in desired media and 1 OD_{600} were harvested by centrifugation at 5000 rpm for 5 min. According to a published protocol (Knop et al. 1999), the pellets were then resuspended in freshly prepared 1 ml of 7.5% β-mercaptoethanol with 2 N NaOH solution. Cells were vortexed and kept on ice for 10 min. Then 200 µl of 55% trichloroacetic acid (TCA) was added and further incubated on ice for 10 min. TCA precipitation was followed by centrifugation at maximum speed for 10 min at 4°C. The supernatant was discarded and again centrifugation was performed for 3 min. Leftover traces of TCA was removed with vaccusip and protein extraction was done in 50 µl HU buffer with 1.5% DTT by heating at 65°C, 10 min, 1400 rpm. After centrifuging it at 14,000 rpm, 5 min, room temperature; 10 µl of the isolated protein lysates were used for immunoblot assays.

3.2.9 Western blot (WB) assays

For immunoblot assays, 1 OD_{600} cells from exponentially growing culture were harvested. Primary cultures were grown in desired media till saturation and then diluted to secondary culture around 0.1 to 0.2 OD_{600}. Total proteins were isolated by TCA precipitation as discussed above. 10 µl of the isolated proteins were loaded on SDS-PAGE and transferred on PVDF membrane for two and half hour at 110 mA; blocked with 5% skimmed milk for 1 hour at room temperature. The membranes were then incubated with primary antibody for 16 hours at 4°C or 3 hours at room temperature, followed by 3x washing for 5 minutes with 1x PBST buffer and incubation of HRP-conjugated secondary antibody for 1 hour at room temperature. Blots were again washed 3x for 5 min with 1x PBST buffer and visualized using chemiluminescence detection reagent from Bio-Rad.

3.2.10 Co-immunoprecipitation (Co-IP) assays

The assay was described previously (Shravan Kumar Mishra et al. 2011).

1. Cells harvesting: 100 OD cells were harvested at log phase OD_{600} around 0.6-0.8 by centrifugation at 3000 rpm for five minutes at 4°C. After centrifugation supernatant was discarded and washed once with 1x PBS. Pellets were re-suspended in cell-lysis buffer containing 1mM PMSF, phosphatase and protease inhibitors and 1% triton X-100 and frozen in liquid nitrogen and stored at -80°C.
2. Cell-lysis: Total cell lysates were prepared by mechanical grinding of frozen pellets with liquid nitrogen in the presence of cell-lysis buffer. The total cell suspension volume was 400 µl. Lysates were pre-cleared two times by centrifugation at 10,000 x g for 10 min at 4°C.
3. Immunoprecipitation (IP): After pre-clearing, the supernatant was transferred to new micro-centrifuge tube and immunoprecipitation was done using appropriate antibody tagged beads (15 µl/50 OD_{600} cells) for 3 hours at 4°C on slow speed rotator. After immunoprecipitation, unbound proteins were washed away by centrifugation at 3000 rpm, 4°C for 2 min, first with 800 µl of wash buffer 1; then three times with wash buffer 2 and finally by lysis buffer (without triton X-100). The supernatant was discarded thoroughly using vaccusip and both inputs (2%), as well as immunoprecipitated proteins, were extracted by heating at 65°C for 10 min in presence of 25 µl HU buffer.

3.2.11 Co-immunoprecipitation (Co-IP) assays (Large Scale)

1. Cells harvesting: Cells were grown to log phase, OD_{600} around 0.8 and total cells corresponding to OD_{600} of 1600 were harvested by centrifugation at 3000 rpm for 10 min at 4°C. After centrifugation, supernatant was discarded and pellets were resuspended in cell-lysis buffer with PMSF, phosphatase and protease inhibitors, snap froze in liquid nitrogen and stored at -80°C. The assay was described previously (Shravan Kumar Mishra et al. 2011).
2. Cell-lysis: Total cell lysates were prepared by mechanical grinding of frozen pellets with liquid nitrogen in the presence of cell-lysis buffer. The total cell suspension volume was 10 ml. Lysates were pre-cleared two times by centrifugation at 10,000 x g for 10 min at 4°C.
3. Immunoprecipitation (IP): After pre-clearing, the supernatant was transferred to a new 15 ml falcon tube and immunoprecipitation was done using appropriate antibody tagged beads (200 µl) for 6 hours at 4°C on slow speed rotator. After immunoprecipitation, unbound proteins were washed away by centrifugation at 3000 rpm, 4°C for 2-3 min, first with diluted lysis buffer; then three times

with wash buffer 1 and finally by wash buffer 2 (without triton X-100). The supernatant was discarded thoroughly using vaccusip and both inputs (~2%), as well as immunoprecipitated 36 proteins, were extracted by heating at 65°C for 10 min in the presence of 25 µl HU buffer. After centrifuging it at 14,000 rpm, 5 min, room temperature; 20 µl of eluted proteins was loaded on NU-PAGE, and coimmunoprecipitated proteins were subjected for mass spectrometry. From the identified proteins in mass spectrometry, we specifically selected all the splicing factors and the table represents the number of unique peptides in mass spectrometry for selected proteins.

3.2.12 Yeast two-hybrid screen

For yeast two-hybrid screening of whole cDNA libraries, potential binding factors were expressed as Gal4 activation domain fusion proteins (AD-fusion), whereas the bait protein carries Gal4 DNA binding domain. In case of physical interaction between the two fusion proteins reporter gene expression (HIS3 and ADE2) was induced which allows growth on selection medium (SC-Ura-Leu-His: 3.5% yeast nitrogen base, 2% glucose and 0.2% amino acid mix). Transformants monitored for interaction on SC-Ura-Leu-His/Ade plates. Positives clones were further retransformed, and interaction is validated by analysis growth on SC-Ura-Leu-His/Ade plates. Assay was described previously in (Varikkapulakkal, Ghosh, and Mishra 2022).

3.2.13 Chromosomal tagging and deletion

Chromosomal tagging and gene deletion was done following published protocols (Janke et al. 2004). The cassette was amplified using long primers that contain sequences of homology to the genomic target location with a mixture of Taq and Vent polymerase using a selection marker cassette. Precipitate the PCR products with 3M sodium acetate (pH 5.3) and 100% ethanol over night at -20℃. Transform competent cells with 10 µl of precipitated product. After transformation, the strains were revived for 16-18 hours on shaking followed by selection on antibiotic plates. The transformants were then screened by immunoblotting assays.

Mating based deletion or tagging was used to generate double mutants or double-tagged strain. Protocol was described in (Ekwall and Thon 2017). Opposite mating type of single mutants or tagged strains were mixed to obtain diploids, spores are checked under microscope at 60x magnification. Once the cells were sporulated, 50-100 µl MQ was added to dilute the cells. 10-12 µl was put on YES plate and was roll down to make a smear. After dissection, plates were incubated at 37°C for 2-3 hours. This will loosen the cell wall around the spore, making it easier to separate the spores during dissection.

Then, they were dissected into individual spores with a dissection microscope. The germinated spores are then screened by selection on different selection plates.

3.2.14 RNA isolation and RT-PCR

RNA isolation and cDNA synthesis were done as described previously (Inada and Pleiss 2010).

1. Cells harvesting: Briefly, 5 OD_{600} cells in logarithmically growing phase were harvested at 30°C (untreated control) or after 15 minutes of heat shock at 37°C by filtration and pellets were stored at -80°C after snap freezing with liquid nitrogen.

2. RNA isolation: Total RNA was isolated by hot acid phenol method using 15 ml phase lock gel heavy tubes. Briefly, pellets were resuspended in acid phenol: chloroform and AES buffer by vortexing. Then the pellets were transferred to 65°C water bath for 7-10 min and vortexed thoroughly once every minute. After lysis, cells were incubated on ice for 5 min and entire organic and aqueous phase was transferred to pre-spun 15 ml phase lock gel tubes. The tubes were centrifuged at 3000 x g at 4°C for 5 min. Then, PCI was added to the gel tubes and again centrifugation was done. Subsequently, chloroform was added to the supernatant and after centrifugation, the aqueous phase was transferred into new 15 ml conical tube with isopropanol and 3 M sodium acetate. The conical tubes were vortexed thoroughly and 2 ml isopropanol slurry was centrifuged at maximum speed for 20 min at 4°C. After centrifugation, the supernatant was discarded and RNA pellets were washed two times with 70% ethanol. The RNA pellets were dried in a vacuum concentrator and finally resuspended in nuclease-free water. It was followed by DNase I treatment for 15 min at room temperature and Zymo-Spin II column was used for clean- up of RNA. Total RNA was quantified with a spectrophotometer by measurement at $OD_{260/280nm}$ (Nanodrop).

3. cDNA synthesis and RT-PCR: cDNA synthesis from 1-2µg total RNA was done using RT and random-hexamer primer at 37°C for 2 hours followed by RT-PCR using target- specific primers and products were analyzed by agarose gel electrophoresis. Primers used in RT-PCR assays of splicing targets are listed in table 3.4.

3.2.15 Ni-NTA pull-down assay

Ubiquitin conjugates were isolated following protocol used in (Thakran et al. 2018). Cells (100 OD_{600}) were harvested at 3000 rpm for 10 min at 4˚C (harvest 1 OD_{600} for TCA-prep as input control). The supernatant was discarded and washed with water at 2500g for 5 mins at 4˚C. Pellets were frozen in liquid nitrogen and stored in -80˚C.

The cells were lysed on ice with 6 ml of 2 N NaOH (45 ml) and 7.5% (v/v) β-mercaptoethanol (5 ml), vortexed, and incubated 15 min on ice. An equal volume of 55% TCA was added, vortexed, and incubated again for 15 mins on ice. The mixture was centrifuge for 15 mins at 2500g at 4°C. The pellet was washed 2x with 5 ml acetone (pre-chilled at -20˚C) for 5 min 2500g at 4°C. The pellet was resuspended with 12 ml buffer A/ 0.05% tween 20 by pipetting up and down with 10 ml pipette. The mixture was transferred in Oakridge centrifuge tube, shaken for 2 hours at room temperature at 15 rpm on rotator wheel. It was then centrifuged for 35 min at 16500g at 4°C. The supernatant was transferred in 15 ml falcon by decanting carefully. 20 mM of imidazole was added. 50 µl Ni-NTA magnetic agarose beads (Qiagen) were added. The sample was kept in rotation for 3 hours at room temperature at10 rpm. A magnetic rack was used to pellet the bead, then the supernatant was sucked off, and the beads were transferred into 1.5 ml MCT. The sample was washed 3x with 800 µl buffer A, 0.05% tween 20, 20 mM imidazole. Then the sample was washed 5x with 800 µl buffer C. 0.05% tween 20. The sample was transferred into a new tube with 100 µl buffer C, put on a magnetic rack, and the supernatant sucked off. The sample was eluted with 30 µl 1% SDS for 10 mins at 65°C. The tube was placed on a magnetic rack to precipitate beads, and elute was transferred to a new tube. The elute was dried in speed vac at 45°C for 25 mins. 25 µl HU buffer was added, and the pellet was denatured at 65°C for 10 mins. The sample was loaded on SDS-PAGE.

Buffer A

6 M Guanidium Chloride	573.24 g
100 mM $NaH_2PO_4.2H_2O$	15.6 g
10 mM Tris	5 ml (2M) /1.21 g
pH	8.0
H_2O	1000 ml

Buffer C

8 M Urea	240.2 g
100 mM $NaH_2PO_4.2H_2O$	7.8 g
10 mM Tris	2.5 ml (2M) /0.606 g
pH	6.3
H_2O	500 ml

3.2.16 Ni-NTA pull-down assay (large scale)

Cells (1000 OD_{600}) were harvested at 3000 rpm for 10 min at 4°C. The supernatant was discarded and washed with water at 2500g for 5 mins at 4˚C. Pellets were frozen in liquid nitrogen and stored in -80˚C.

The cells were lysed on ice with 20 ml of 2 N NaOH (45 ml) and 7.5% (v/v) β-mercaptoethanol (10 ml), vortexed, and incubated 15 min on ice. An equal volume of 55% TCA was added, vortexed, and incubated again for 15 mins on ice. The mixture was centrifuge for 15 mins at 2500g at 4°C. The pellet was washed 2x with 5 ml acetone (pre-chilled at -20˚C) for 5 min 2500g at 4˚C. The pellet was resuspended with 30 ml buffer A/ 0.05% tween 20 by pipetting up and down with 10 ml pipette. The mixture was transferred in Oakridge centrifuge tube, shaken for 2 hours at room temperature at 15 rpm on rotator wheel. It was then centrifuged for 35 min at 16500g at 4°C. The supernatant was transferred in 50 ml falcon by decanting carefully. 20 mM of imidazole was added. 240 µl Ni-NTA magnetic agarose beads (Qiagen) were added. The sample was kept in rotation for 3 hours at room temperature at 10 rpm. A magnetic rack was used to pellet the bead, the supernatant was sucked off, and the beads were transferred into 1.5 ml MCT. The sample was washed 3x with 800 µl buffer A, 0.05% tween 20, 20 mM imidazole. Then the sample was washed 5x with 800 µl buffer C. 0.05% tween 20. The sample was transferred into a new tube with 100 µl buffer C, put on a magnetic rack, and the supernatant sucked off. The sample was eluted with 100 µl 1% SDS for 10 mins at 65°C. The tube was placed on a magnetic rack to remove beads, and elute was transferred to a new tube. The elute was dried in speed vac at 45°C for 25 mins. 50 µl HU buffer was added, and the pellet was denatured at 65°C for 10 mins. The sample was loaded on Nu-PAGE and proteins were subjected for mass spectrometry.

3.2.17 Membrane extraction protocol

The protocol was adapted from (Arines and Li 2022). 7 OD_{600} cells of logarithmically growing cultures were harvested and frozen in liquid nitrogen. 1.2 ml of 10% TCA was added and kept in ice for 30 minutes. They were centrifuged at 13500g for 15 minutes and the supernatant was removed. The pellets were washed with 500 µl of 0.1% TCA. Cells were lysed using 70 µl of 2x boiling buffer and 50 µl of acid washed glass beads. This mixture was placed in a bead beater for 5 minutes at maximum speed. Then the samples were incubated at 42 ˚C for 5 minutes. Then 70 µl of urea sample buffer was added and again kept in bead beater for 5 minutes at maximum speed. Then the samples were again incubated at 42°C for 5 minutes. Finally, it was centrifuged at 13500g for 15 minutes and 10 µl of the supernatant was loaded.

Boiling Buffer (2x)

Reagent	Final concentration
Tris, pH 7.5	50 mM
EDTA, pH 8.0	10 mM
Dithiothreitol (DTT)	200 mM
SDS	4% w/v
Urea	6 M

Urea Sample Buffer (2x)

Reagent	Final concentration
Tris, pH 6.8	150 mM
Urea	6 M
Glycerol	10% v/v
SDS	4% w/v
Bromophenol Blue	0.01% w/v

3.2.18 Microscopy

S. pombe cultures were grown in EMM media at 30°C with shaking for 24-36 hours and then reinoculated in fresh EMM media to an OD_{600} around 0.2. Cells were incubated at 30°C with shaking at 220 rpm till the OD_{600} nearly 0.5–0.7 and plated on 1.5% agarose slabs made with EMM minimal media and imaged using glass-bottom confocal dishes (35 mm, SPL life sciences). The agarose slab were prepared as described in (Chimthanawala and Badrinarayanan 2019). For low glucose treatment, the cells growing in mid-log phase (OD_{600} 0.5–0.7) were pelleted down by centrifugation at 3000g for 5 min, washed with sterile water and then transferred to fresh EMM media with 4.4 mM glucose for 6 hours and imaged. Imaging was carried out using a wide-field epifluorescence microscope (Eclipse Ti-2E, Nikon) with a 63× oil immersion objective, illumination from a pE4000 light source (CoolLED), and Hamamatsu Orca Flash 4.0 camera.

For quantification of intracellular/peripheral fluorescence signals, the protocol was adapted from (Hovsepian et al. 2018). A first ellipse was drawn around each cell (n > 50 cells) using Fiji (ImageJ2), and a second ellipse was drawn inside the cell so as to exclude the plasma-membrane localized signal. The integrated density (IntDens) of both of these regions of interest were measured, to which the back-ground noise for each ellipse was subtracted, giving a corrected value of IntDens (IntDensCorr). The difference (IntDensCorr[1st ellipse]−IntDensCorr[2nd ellipse]) was taken as a value of the plasma membrane signal. An unpaired, two-tailed t test was performed and the p values are indicated (NS: $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

3.2.19 Bioinformatics of *S. pombe* introns

The Sde2-dependent introns were obtained from the splicing-sensitive microarray of the *Δsde2* strain (GEO accession number GSE97097) (Thakran et al. 2018). Nucleotide sequences were obtained from the PomBase server. RNA structures were predicted on the RNAfold WebServer (Gruber et al. 2008). For intron analysis, the complete dataset of introns was downloaded from PomBase and then mapped with the microarray data. A cut-off of 0.5 $\log_2$*Δsde2/*wt ratio (~1.4-fold difference) of intron-retention values obtained with the strains grown at 30°C was applied. Introns with ratios >0.5 were considered Sde2 dependent, and the remaining introns were considered Sde2 independent. The database was then matched with the following list of BP sequences: ‘CCAAC’, ‘CUAAC’, ‘UUAAC’, ‘UCAAC’, ‘CCGAC’, ‘CUGAC’, ‘UUGAC’, ‘UCGAC’, ‘CCAAU’, ‘CUAAU’, ‘UUAAU’, ‘UCAAU’, ‘CCGAU’, ‘CUGAU’, ‘UUGAU’, ‘UCGAU’, ‘GUAAC’, ‘CUCAC’, ‘AUAAC’, ‘UUGAC’, ‘CUGAU’, ‘CUAAA’, ‘CUUAC’ and ‘CUAAG’. For both Sde2-dependent and -independent introns, the distances of BP sequence (closest to the 3′ss ‘NAG’) from the 5′ and 3′ splice sites were calculated by counting the number of bases in between, and frequency distributions were computed for comparative analysis. Polypyrimidine tracts were defined as at least six consecutive non-adenine nucleotides containing no fewer than three uridines (Kupfer et al. 2004) and were identified using an in-house-written script. For sequence logos, the nucleotides spanning the desired window around the 5′ss, BP nucleotides and 3′ss for Sde2-dependent and -independent introns were obtained using custom scripts in R. Sequence logos were then created by using the ggseqlogo package in R (Wagih 2017). Violin plots were generated using the ggplot2 package in R.

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PUBLICATIONS

1. Anil,A.T.*, Choudhary,K.*, **Pandian,R**.*, Gupta,P., Thakran,P., Singh,A., Sharma,M. and Mishra,S.K. (2022) Splicing of branchpoint-distant exons is promoted by Cactin, Tls1 and the ubiquitin-fold-activated Sde2. *Nucleic Acids Res.*, **50**, 10000–10014. * - co-first authors.

2. Sajeevan,A., **Pandian,R**. and Mishra,S.K. (2022) Vectors with a flexible multiple cloning site and modular epitope tags for gene expression studies in Schizosaccharomyces pombe. *Gene Reports*, **29**, 101681.