Declaration

I hereby declare that I alone composed this thesis and that work presented here is my own, except where stated otherwise.

Tania Auchynnikava
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First I would like to thank people without whom my PhD here would not be possible. My supervisor Jean, for having me in her lab, providing so much needed support in both a scientific and human way. I would like to express my gratitude to Sir Kenneth Murray for funding my PhD, and to my undergraduate supervisor, Dr Evgeniy Nickolaichik, who literally forced me to apply for the studentship.

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CONTENTS

CONTENTS.................................................................1
ABBREVIATIONS.......................................................3

CHAPTER 1........................................................................4
1.1 INTRODUCTION TO DExD/H BOX HELICASES..............4
1.5 FUNCTIONS OF DExD/H BOX HELICASES..................11
1.6 CO-FACTORS OF DExD/H BOX HELICASES...............11
1.7 POSSIBLE TARGETS FOR DExD/H-BOX HELICASES IN PRE-MRNA SPlicing AND PRE-RNA PROCESSING.....15
1.2.1 INTRODUCTION TO SPlicing..................................20
1.2.2 SEQUENCES IMPORTANT FOR SPlicing..................21
1.2.3 PRE-MRNA SPlicing REACTION..........................22
1.2.4 THE SPliceosome...............................................23
1.2.5 SPliceosome ASSEMBLY......................................24
1.2.6 TOWARDS A CATALYTIC SPliceosome...................26
1.2.7 THE CATALYTIC SPliceosome................................29
1.2.8 THE POST-SPliceosome.......................................30
1.2.9 DExD/H BOX PROTEINS AS FIDELITY FACTORS.......31
1.3.1 RIBOSOMAL BIOSYNTHESIS................................33
1.4 CROSSALK............................................................38

AIMS OF THE PROJECT..................................................41

CHAPTER 2........................................................................42
MATERIALS AND METHODS..........................................42
2.1.1 ENZYMES AND CHEMICALS.................................42
2.1.2 COMMONLY USED BUFFERS..................................42
2.2 CULTURE AND MEDIA............................................42
2.3 BACTERIAL STRAINS...............................................43
2.4 YEAST STRAINS.....................................................44
2.5 PLASMIDS..............................................................44
2.6 Oligonucleotides.....................................................45
2.7 ANTISERA..............................................................46
2.8 BACTERIAL TECHNIQUES.........................................47
2.9 YEAST TRANSFORMATION.........................................47
2.10 RECOMBINANT DNA TECHNIQUES.........................47
2.11 POLYMERASE CHAIN REACTION............................48
2.12 RNA TECHNIQUES..................................................48
2.12.1 RNA EXTRACTION............................................48
2.12.2 IN VITRO TRANSCRIPTION..................................49
2.12.3 IN VITRO SPlicing.............................................50
2.12.4 CO-IMMUNOPRECIPITATION OF snRNAs/SPliceosome.51
2.12.5 EXTRACT PREPARATION FOR RIBOSOMAL RNA PROCESSING IMMUNO-PRECIPITATIONS..........53
2.12.6 RNA GEL ELECTROPHORESIS AND NORTHERN BLOT ANALYSIS........................................54
2.12.7 PULSE CHASE ANALYSIS....................................55
2.13 PROTEIN METHODS...............................................55
2.13.1 PURIFICATION OF PRP43 FROM E. COLI...............55
2.13.2 YEAST SPlicing EXTRACT....................................56
2.13.3 CO-IMMUNOPRECIPITATION OF PROTEINS FROM YEAST PROTEIN EXTRACTS......................57
2.13.4 PREPARATION OF TOTAL PROTEIN EXTRACTS FOR WESTERN IMMUNOBLOTTs....................58
2.13.5 GLYCEROL GRADIENT ANALYSIS........................58
2.14 SPlicing ANALYSIS BY REAL-TIME QUANTATIVE RT-PCR..........................58
2.15 IMMUNOFLUORESCENT STAINING..........................61

CHAPTER 3........................................................................62
ANALYSIS OF NTR COMPLEX FUNCTION IN SPlicing...........62
3.1 INTRODUCTION .................................................................................................................. 62
3.2 DEPLETION OF SPP382P AFFECTS SPLICEOSOME DISASSEMBLY ........................................... 64
3.3 NTR2 PROTEIN IS ASSOCIATED WITH THE POST-SPLICING COMPLEX ........................................ 66
3.4 Spp382p and Ntr2p CO-sediment in Glycerol Gradients ............................................................... 70
3.6 SPP382P IS NECESSARY FOR PRP43P ASSOCIATION WITH THE SPLICEOSOME ....................... 71
3.7 SPP382P, NTR2P AND PRP43P ARE ASSOCIATED WITH CATALYTIC SPLICEOSOMES ............... 74
3.8 DISCUSSION .......................................................................................................................... 77

CHAPTER 4 .................................................................................................................................... 79
SPP382 IS A PRE-rRNA PROCESSING FACTOR .............................................................................. 79

CHAPTER 5 ........................................................................................................................................ 88
ROLE OF PRP43 PROTEIN IN SPlicing .......................................................................................... 88
5.2 PRP43 PROTEIN IS ASSOCIATED WITH COMPLEX A ..................................................................... 89
5.3 ROLE OF THE C-TERMINUS OF PRP43p IN SPlicing AND PRE-rRNA PROCESSING .................. 95
5.4 PRP43 IS NECESSARY FOR U6 snRNA RELEASE FROM THE POST-SPLICING COMPLEX .......... 99

CHAPTER 6 ........................................................................................................................................... 105
PRP43p requires SPP382p for PRE-rRNA PROCESSING ................................................................. 105
6.2 SPP382p is an ACCESSORY FACTOR FOR PRP43p IN PRE-rRNA PROCESSING ....................... 106
6.3 G-PATCH OF SPP382P RESCUES GROWTH DEFECT CAUSED BY DEPLETION OF SPP382P ................ 108
6.4 OVER-EXPRESSION OF SPP382P CAUSES DELOCALISATION OF PRP43p ............................... 115
6.4 DISCUSSION .................................................................................................................................. 117

CHAPTER 7 .......................................................................................................................................... 119
7 final DISCUSSION AND FUTURE EXPERIMENTS ........................................................................ 119
7.2 THE ROLE OF PRP43p IN PRE-rRNA PROCESSING ..................................................................... 120
7.3 CROSSTALK .................................................................................................................................. 121

BIBLIOGRAPHY ............................................................................................................................. 123
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>aa</td>
<td>Amino acid(s)</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base-pair</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DTT</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ETS</td>
<td>Externally transcribed spacer</td>
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<td>g</td>
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<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>L</td>
<td>Litres</td>
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<td>Luria broth</td>
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<tr>
<td>mRNA</td>
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<tr>
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<tr>
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<td>Nucleotides</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>NTP</td>
<td>Nucleotide triphosphate</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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</tr>
<tr>
<td>r-protein</td>
<td>Ribosomal protein</td>
</tr>
<tr>
<td>RT</td>
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</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>SDS</td>
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<td>Single stranded DNA</td>
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Chapter 1

1.1 Introduction to DExD/H box helicases.
DExD/H-box proteins are a class of enzymes that are thought to unwind RNA-RNA duplexes in an NTP-dependent manner, and therefore are required for all aspects of RNA metabolism (Bork and Koonin, 1993; Koonin, 1993). All DExD/H-box proteins employed in splicing and pre-rRNA processing belong to the SF2 family of helicases, and can be easily identified by the presence of 7-9 conserved motifs (de la Cruz et al., 1999). An amino acid sequence in motif II gives the name for the whole family (Linder et al., 1989). Mutational analysis implicated these motifs in RNA binding and translocation (motifs Ia, Ib, IV and V), NTP binding and hydrolysis (I, II, VI and Q) and motif III is believed to couple NTP hydrolysis to remodelling events reviewed in Tanner and Linder, 2001 (figure 1.1).

Figure 1.1. Schematic representation of DExD/H helicases structure. The conserved motifs of DExD/H box helicases are folded into two distinct domains (highlighted). Role of the motifs in substrate recognition and ATP binding is shown (adapted from Jankowsky et al., 2001).

Among RNA helicases of the SF2 superfamily, three subfamilies can be distinguished: DEAD, DEAH and Ski2-like (DExH) proteins, which are often referred by the common name DExD/H-box helicases (Cordin et al., 2006; de la Cruz et al., 1999; Tanner and Linder, 2001). Despite sharing a highly similar structure, several distinctive characteristics allowed identification of these sub-
families. Thus, DEAD-box proteins are non-processive RNA binding ATPases, whose main function is believed to be displacing proteins from RNA (Cordin et al., 2006; Linder et al., 2001). DEAH-box proteins are NTPases, which show significant levels of NTP hydrolysis even without RNA bound. The third sub-family, DExH-box helicases, are ATP-dependent and processive enzymes that are capable of active translocation along the substrate (Fairman et al., 2004; Kawaoka et al., 2004). The mechanism of their translocation is believed to be similar to that of processive DNA helicases (Pyle, 2008).

Structurally, all DExD/H-box helicases are very similar and possess two RecA-like domains connected by a linker (Caruthers and McKay, 2002). It is believed that this linker enables relative movements of domains during different stages of the biochemical cycle of the helicases. However there is a noticeable difference in the domain organisation between the subfamilies, which is believed to influence their biochemical properties (figure 1.2)(Pyle, 2008). Structural studies of DEAD-box proteins revealed that their two RecA-like domains are connected with a very flexible linker that allows significant movement of helicase domains relative to each other dependent on their RNA and ATP-nucleotide bound state (Pyle, 2008). At the same time, helicase domains of DExH-box proteins are positioned close to each other at all times even without bound nucleotide, which determines the ability of the proteins of this group to hydrolyse ATP even in the absence of RNA (Pyle, 2008).
Despite high similarity in sequence and structure, DExD/H-box helicases possess high specificity in vivo. The mechanisms, which govern their specificity, activity and recruitment to the specific substrates, remain largely unknown. It is believed that specificity and activity of DExD/H-box proteins is mostly determined by the protein-protein interactions within large protein complexes (Silverman et al., 2003). As the C- and N-termini of DExD/H-box helicases are variable in sequence and composition, it is suggested that they are largely responsible for the specificity of interactions of DExD/H box proteins with either co-factors, or substrates (Edwalds-Gilbert et al., 2004; Silverman et al., 2003). Several examples of the role of flanking regions in the regulation of activity and specificity of DExD/H-box proteins have been obtained. The C-terminal domain of E. coli DEAD-box helicase DpbA enables it to recognize a specific part of 23S rRNA, which also provides a unique example of sequence specificity among DExD/H-box helicases (Fuller-Pace et al., 1993; Pugh et
Over-expression of the flanking regions of yeast DEAH-box helicase Prp16p impairs viability, suggesting that flanking regions have a dominant negative phenotype, and possibly play a role in binding of the Prp16p to the spliceosome (Burgess et al., 1990; Wang and Guthrie, 1998). Also the N-terminus of Prp2p was shown to be necessary for the interaction with Spp2p, which is required for Prp2p recruitment to the spliceosome (Edwalds-Gilbert et al., 2004; Silverman et al., 2004).

However, eIF4AIIIp and Dbp8p, which do not possess extensive flanking sequences, were shown to be able to bind their co-factors with the conserved helicase core; this suggests that flanking regions, while important for the specificity, are not the only regions capable of interaction with co-factors (Granneman et al., 2006b; Tanner and Linder, 2001).

Several biochemical characteristics of DExD/H-box helicases are important for their function.

1.2 Nucleic acid binding.
DExD/H-box helicases are thought to unwind RNA-RNA duplexes, therefore they should have the capacity to bind RNA. For several DExD/H-box proteins no RNA binding activity has been shown; it is largely believed that their RNA binding activity depends on co-factors, which are absent in vitro assay (Silverman et al., 2003). However, there is a principal difference between RNA binding between DEAD and DExH helicases. DEAD-box proteins require RNA binding for their ATPase activity, while DExH-box proteins are able to hydrolyze ATP even without RNA present (Lost et al., 1999; Tanaka and Schwer, 2005, 2006). Most DExD/H box helicases show no sequence specificity in vitro (apart from bacterial DEAD-box helicase DbpA mentioned above), and therefore the high specificity of DExD/H box helicases is believed to depend on multiple protein-protein interactions (Silverman et al., 2003).
1.3 Activity of DExD/H helicases.

RNA helicases are thought to unwind RNA duplexes in an NTP-dependent manner. Two principally different mechanisms of activity for DEAD and DExH proteins were proposed based on crystal structures and their biochemical properties (figure 1.2).

Without ATP and RNA bound, DEAD-box helicases adopt various "open" conformations with no contacts between two domains as was revealed after solving their crystal structures (reviewed in Cordin et al., 2005). Cooperative binding of ATP and RNA brings the two domains together, forming a "closed" conformation with extensive interactions between the domains as was revealed by crystal structures of DEAD-box helicase Vasa and eIF4A with a non-hydrolysable analog of ATP and ssRNA (Bono et al., 2006; Sengoku et al., 2006). It has also been suggested that the change from "open" to "closed" conformations activates DEAD-box proteins enabling them to perform ATP hydrolysis and unwinding. Recently, a FRET assay with Bacillus subtilis DEAD-box helicase YxiN carrying donor and acceptor on different domains showed that indeed cooperative binding of ATP and ssRNA causes changes in conformation of YxiN; ATP hydrolysis leads to "open" conformation, supporting the existence of a biochemical cycle of "open"-"closed" conformations (Theissen et al., 2008). In vitro studies established that the unwinding step of DEAD-box proteins is only 5-6 bp, which is approximately the binding site of a single molecule of the enzyme, and it is believed that DEAD-box helicases couple binding of RNA to unwinding of RNA-RNA duplex. It was suggested that DEAD-box enzymes upon their binding of RNA duplex, change their conformation in ATP-dependent manner thus they destabilize the duplex, releasing ssRNA. Subsequent change of conformation probably leads to spontaneous dissociation of the helicase from RNA (Pyle, 2008).

Processive DExH helicases load on the ss overhang, and are able to translocate alongside the loading strand displacing RNA or proteins, therefore the best way to describe their activity mode as "wire strippers" (Pyle, 2008).
1.4 Translocation.
Several models originally developed for DNA and processive RNA helicases, based on observation in vitro, are likely to be applicable to explain the mode of action of DExH helicases. The models can be divided into two large groups: deterministic stepping models and stochastic brownian models, which in a certain sense are complementary to each other. Both of these models rely on the fact that a helicase has at least two nucleic acid binding sites, which independently bind and release the substrate with one binding site at a time staying bound to the substrate. Stepping models include the active rolling model and the inchworm model (reviewed in Soultnanas and Wigley, 2000, 2001; Tanner and Linder, 2001). The active rolling model is applicable to dimeric helicases, and suggests that the two nucleic acid binding sites have high affinity to either dsRNA or ssRNA, respectively. ATP hydrolysis causes changes in the affinity state, and results in duplex unwinding. The inchworm mechanism can be used to describe ATP-hydrolysis dependent relative movements of domain 1 and domain 2 within a helicase monomer. According to this model, the substrate is bound simultaneously by two domains, but only one of them is tightly bound to the substrate at any time. To date the inchworm model is the best to explain DExD/H-box helicase activity.
The active rolling model requires a dimer for the activity in which each monomer has a different affinity to the substrate. These conformations require binding ATP and ATP hydrolysis. The inchworm model works for monomeric or oligomeric helicases. In this case distances between domains vary with ATP binding and hydrolysis. (Adapted from Tanner and Linder, 2001)

The Brownian model is also a two-state model, but instead of deterministic movement it invokes Brownian motion and two different states of helicase: with high and low affinities to the substrate based on NTP bound states (Levin et al., 2005). When the helicase changes its nucleotide bound state, its affinity to the nucleic acid changes (as in case of the inchworm model). It can move alongside the substrate in either direction in Brownian motion or dissociate. After resuming the high affinity state, the helicase makes a move forward, performing the unwinding (Levin et al., 2005). This model agrees well with low processivity of RNA helicases, however the stochastic nature of the model does not agree with the observed polarity, and does not really agree with a proposed role of "motors" for DExD/H-box helicases.
1.5 Functions of DExD/H box helicases.
Despite that DExD/H proteins are called RNA helicases, and *a priori* are able to unwind RNA duplexes, increased evidence is accumulating regarding functions of these enzymes in activities unrelated to duplex unwinding. Additionally, many proteins of this class show very weak unwinding activity *in vitro*, which initially was attributed to lack of co-factors in *in vitro* assays. However, at present DEAD-box proteins viewed as RNA binding proteins, which are unable to translocation, and the primary function for DEAD-box proteins is thought to be protein displacement (Cordin et al., 2006; Fairman et al., 2004). This function for DEAD proteins was supported not only in *in vitro* studies, but also in analyses of *in vivo* genetic interactions. If developed further, this idea suggests the function of DEAD-box proteins could assist in snRNP maturation and biogenesis through prevention of premature interactions, or facilitating processing events by displacing proteins from the processing sites. Recently, the functions of Ded1p and Mss116p in strand annealing were uncovered (Halls et al., 2007; Yang and Jankowsky, 2005). Indeed, these proteins were shown to stabilize RNA duplexes in the absence of ATP, suggesting that DExD/H box proteins might play opposite roles in RNA processing. For some processive helicases duplex unwinding is a confirmed function (Raghunathan and Guthrie, 1998), however they are also shown to be able to remove proteins from the single stranded RNA (Linder et al., 2001).

1.6 Co-factors of DExD/H box helicases.
It has been suggested that the activity and specificity of DExD/H box proteins is largely modulated by protein-protein interaction, and that they are required to be part of a complex to be active. Numerous co-factors for the DExD/H box proteins were identified. Some of them are required for the association with complexes, and others were shown to be required for the activity. Both of these functions might be performed by the same protein, however most of the studies assayed only one or the other. Several examples of helicases and their co-factors are given below.
Several co-factors, enhancing activity of DExD/H-box helicases were identified. Interestingly, some of the co-factors have RNA binding motifs, or putative RNA binding motifs. As many DExD/H box helicases were shown to have weak RNA binding, it is possible that using an RNA-binding protein as a cofactor is a way of stimulating the helicase activity (Silverman et al., 2003). It is conceivable that interacting proteins help to "correct" conformation of DExD/H box helicases, and switch helicase domains into a functional mode. Alternatively, the RNA binding domains of the accessory proteins might provide a bridge between DExD/H box helicase and RNA substrate (Silverman et al., 2003).

Studies of DEAD-box helicase RhlB of *E. coli* provided support for the first hypothesis. RhlBp is a component of the degradosome, which contains RNAseE (Py et al., 1996). RhlB was shown to interact directly with RNAseE, and this interaction is required for the stimulation of activity of RhlB. Additionally, it was established that small recombinant peptides of RNAseE, which mimicked RNAseE interaction with RhlB, were able to stimulate ATPase activity of RhlB, and this implied that the RNA-binding domain of RNAseE was not required for the activity of RhlB (Vanzo et al., 1998). It was therefore proposed that interaction between these two proteins triggers small structural changes in conformation of RhlB, which lead to its activation and eliminated the possibility that RNAseE acts like a bridging factor (Chandran et al., 2007).

Another well-known example is the translation initiation eIF4F complex from higher eukaryotes (reviewed in (Gingras et al., 1999). One of the components of this complex, DExd/H box helicase eIF4AIII was stimulated by protein eIF4G (Rogers et al., 2001). A co-crystal structure of eIF4AIII and eIF4G showed that eIF4G interacts with both RecA-like domains of eIF4AIII; eIF4G was proposed to act as a "stopper", preventing eIF4AIII from going into a non-productive conformation (Marcotrigiano et al., 1997).
The only known example of helicase activator in pre-rRNA processing in yeast is Esf2p, which is required for activation of helicase Dbp8p (Granneman et al., 2006b). *In vitro* experiments showed that the presence of Esf2p and RNA stimulated the ATPase activity of Dpb8p, by increasing its affinity for ATP (Granneman et al., 2006b), providing an example of modulation of biochemical parameters of helicase by co-factor.

DEAD-box helicase Dbp5p involved in mRNA export provides an interesting example of helicase regulation (Snay-Hodge et al., 1998; Weirich et al., 2006). Dbp5p, a cytoplasmic helicase, interacts directly with the nuclear pore protein Gle1p. Interestingly, it was shown that Dbp5p does not show helicase activity or RNA binding activity *in vitro* when assayed alone. However, addition of Gle1p increases the biochemical activity of Dbp5p, suggesting that Gle1p is an activator of Dbp5p. As these proteins have different sites of localization, it was proposed that Gle1p locally activates Dbp5p thus providing an example of means of regulation of DExD/H-box helicases by separation of helicase in space from its activators to prevent premature action (Weirich et al., 2006).

Prp43 protein and its co-factors are the object of the research in this study, and will be discussed in detail below.

Several other studies showed genetic interactions of helicases with RNA binding proteins, however no biochemical studies were performed. Thus, DEAD-box helicase Prp5p involved in spliceosome assembly has a genetic interaction with RNA-binding protein Cus2p (Perriman et al., 2003), and DEAH-box protein Prp2p requires G-patch protein Spp2p for association with the spliceosome (Silverman et al., 2004). In pre-rRNA processing, proteins Rrp5p and Gar1p were shown to have genetic interaction with helicase Rok1p with pre-rRNA processing machinery (dosage rescue and synthetic lethality, respectively) (Torchet et al., 1998; Venema and Tollervey, 1996). Remarkably, several helicases were found to possess genetic interactions with each other. Thus, in a yeast two-hybrid assay, splicing DEIH-box helicase Brr2p
interacted with DEAH-box proteins Prp2p and Prp16p, and Prp16p interacted with Prp22p (van Nues and Beggs, 2001). Moreover, Prp22p seems to require Prp16p for its association with the spliceosome (McPheeters et al., 2000). In pre-rRNA processing, weak yeast two-hybrid interaction between Dpb6p and Dpb9p, and synthetic growth defect between Dpb7p and Dpb3p were reported (Bernstein et al., 2006; Daugeron et al., 2001; Granneman et al., 2006a). Also, it has been shown that increased dosage of Dpb9p suppresses mutations in DBP6 (dbp6-4, dbp6-13), suggesting the existence of a functional relationship between the two proteins (Daugeron et al., 2001). Additionally, synthetic enhancement of the defect caused by mutations in DBP6, DBP7 and DBP9 were reported, which indicates that these helicases are likely to affect the same pathway (Daugeron et al., 2001). The possibility of close association between helicases was also proposed by Bernstein and colleagues as their investigation of DExD/H-box helicases revealed that some of them co-precipitate other helicases which were shown to be functionally close in pre-rRNA processing (Bernstein et al., 2006). Strikingly, a gene dosage effect was observed for pre-rRNA processing helicases in E.coli as over-expression of the DExD/H-box helicase RhlE, participating in pre-rRNA processing, suppresses or exacerbates the slow growth phenotypes of mutations in helicases CsdA and SrmB, respectively (Jain, 2008). This allows speculation that interactions between helicases are conserved through evolution and different RNA processing machineries, and likely has some functional significance. Also it is possible that there might be an as yet unappreciated level of regulation between helicases. As many processive viral RNA helicases were shown to act as oligomers, it is conceivable that association between DExD/H-box helicases serves as some kind of activation and regulation mechanism. Moreover, the existence of such a network of interactions supports the idea that DExD/H-box proteins might act as driving motors for biological processes, possibly by chain activation of helicases.
Possible targets for DExD/H-box helicases in pre-mRNA splicing and pre-rRNA processing

At present, the targets of most DExD/H box helicases remain unknown; although, several genetic and crosslinking studies have identified potential targets for helicases or RNPase activity of DExD/H box helicases.

Potential targets of DExD/H-box helicases in splicing.

In splicing in many cases potential targets of DExD/H-box helicases were characterised (figure 1.4) Through the use of yeast genetics it was established that altering certain RNA-protein interactions could make DEAD box proteins dispensable for growth. Thus, during spliceosome assembly the need for the essential Prp28p can be alleviated when either U1-Cp or the U1 snRNA structure are altered (Chen et al., 2001; Strauss and Guthrie, 1994). Analysis of several mutants suggested that destabilization of interaction of U1 with 5'SS and altering the structure of U1-Cp probably makes the switch from U1/5'SS to U6/5'SS easier to occur as U1-Cp is believed to stabilize the U1 association with the 5'SS. Therefore it was proposed that Prp28p might play a role in displacing U1-Cp from the 5'SS (Chen et al., 2001).

At another step of spliceosome assembly BBP and Mud2 proteins should be displaced from the branch-point region by U2 binding to the 3'SS. Two DEAD-box helicases were implicated in this step of spliceosome assembly – Sub2p and Prp5p. It was established that deletion of the non-essential MUD2 gene makes otherwise essential Sub2p helicase dispensable for this stage of spliceosome assembly (Kistler and Guthrie, 2001). Additionally, deleting the non-essential RNA binding protein Cus2p alleviates requirement for the activity of Prp5p (Perriman and Ares, 2000), suggesting that Prp5p might displace Cus2p from U2. However, ATPase activity of Prp5p has been shown to be required for U2 association with the branch-point region (Xu and Query, 2007), however these two functions might be overlapping.

While DEAD-box helicases were implicated in protein displacement, the precise roles of DEAH-box helicases remains largely unidentified. Recently developed
knowledge about catalytic reactions identified a putative role for Prp16p in remodeling of the U2 snRNA stem IIc to IIa, therefore playing a role in toggling of U2 snRNA structure between two conformations (Perriman and Ares, 2007). Additionally, cross-linking data revealed interaction of Prp16p with the 3'SS, suggesting a possible role for Prp16p in remodeling of the 3'SS during catalytic steps (McPheeters and Muhlenkamp, 2003). Mutational analysis implicated another DEAH-box helicase Prp22p in mRNA release from the spliceosome, and recently direct evidence for this was obtained (Wagner et al., 1998). Cross-linking studies and an RNase H protection assay found Prp22p to be associated with the 3'SS, which supported a function for Prp22 in unwinding of mRNA/U5 loop contacts and thus promoting mRNA release (Schwer, 2008).

The only DEIH helicase of the spliceosome, Brr2p, is also the only integral helicase of the spliceosome. As was stated above, the main distinction of the proteins belonging to this class is their processivity. Indeed, Brr2p was implicated in unwinding U4/U6 duplex during the formation of the catalytic spliceosome (Raghunathan and Guthrie, 1998). Furthermore, the helicase activity of Brr2p was implicated in spliceosome disassembly (Small et al., 2006), and it was shown that Brr2p unwinds U2/U6 duplex in vitro (Xu et al., 1996), suggesting that Brr2p might play an opposite roles during spliceosomal cycle.

Prp2p and Prp43p are helicases with unknown RNA/protein targets, despite it is known that Prp2p is involved in activation of the spliceosome, and Prp43p plays a role in spliceosome disassembly.
Figure 1.3 RNA helicases in pre-mRNA splicing and schematic representation of pre-mRNA splicing cycle. DExD/H-box helicases involved at each step of splicing reaction are indicated. (Adapted from (Bleichert and Baserga, 2007).

Potential targets of DExD/H-box helicases in pre-rRNA processing.
Pre-rRNA processing, like splicing, requires RNA-RNA remodelling, and 18 DExD/H-box helicases were found to play a role in ribosomal RNA maturation (figure 1.4). The helicases of pre-rRNA processing mostly belong to the DEAD-box subfamily, which are non-procesive enzymes. There are only three exceptions - Dhr1p and Dhr2p are classified as DEAH-box class proteins, and Mtr4p is a DExH-helicase.
Figure 1.4 Schematic representation of DExD/H-box helicases involved in pre-rRNA processing pathway. Simplified schematic of pre-rRNA processing for small (18S; left side) and large subunit (5.8S, 25S; right side) maturation with indicated cleavage sites. The 18S, 5.8S and 25S are transcribed as a polycystronic precursor. Several endo- and exonucleolytic cleavage steps occur in order to mature the rRNAs. RNA helicases involved in 18S maturation are indicated in red, those involved in 5.8S and 25S maturation are indicated in green, and RNA helicases that have been implicated in biogenesis of both large and small ribosomal subunits are represented in yellow. Esf2p, the only known and characterised co-factor of a helicase involved in ribosome biogenesis, is indicated in blue. (Adapted from Bleichert and Baserga, 2007).

The functions of the helicases in pre-rRNA processing remain largely unknown, apart from several examples given below. It was proposed that the role of DExD/H-box helicases might be remodeling multiple snoRNA-rRNA interactions. However, the only snoRNAs directly implicated in cleavages (U3, U14 and snR30) are essential. The rest of the snoRNAs are dispensable for growth, and even double deletion mutants do not show a phenotype. Therefore it is unlikely that the sole
function of DExD/H box helicases in pre-rRNA processing is snoRNA remodelling. At the same time, experiments have shown that depletion of Dbp4p results in retention of U14 and Snr41 snoRNAs with pre-rRNA processing particles, and led to the proposition that Dbp4p unwinds U14 snoRNA from pre-rRNA processing machinery (Kos and Tollervey, 2005). Direct evidence of the unwinding has not been obtained, however, the existence of a genetic interaction between U14 and Dbp4p suggests that U14 retention is likely to be a direct consequence of depletion of Dbp4p (Kos and Tollervey, 2005; Liang et al., 1997). Additionally, a mutation in Roklp helicase is synthetic lethal with snR10, suggesting that Roklp might be involved in remodelling of snoRNA interaction with pre-rRNA (Venema et al., 1997). Strikingly, depletion of Haslp resulted in retention of U14, U3, snR3, snR10, snR30, snR63 and snR77 with pre-rRNA processing particles, suggesting that either Haslp and Dbp4p have overlapping functions, or retention of snoRNAs might be an indirect result of faulty pre-rRNA processing (Liang and Fournier, 2006). Additionally, depletion of several other helicases (Dbp8p, Roklp, Dhrlp, Dhr2p) has no effect on snoRNA association with the pre-rRNA processing machinery (Kos and Tollervey, 2005), indicating that remodeling of snoRNA - pre-rRNA interactions is not the only function of the helicases. Therefore, despite that a direct effect of DExD/H-box helicases on snoRNA remodeling is possible, it is likely that helicases in pre-rRNA processing perform other unidentified functions.

Attempts were made to address possible functions by over-expressing dominant negative mutants of DExD/H-box helicases and assaying pre-rRNA processing defects, however, no specific phenotype was attributed to particular helicases and little information about their function was revealed (Bernstein et al., 2006; Granneman et al., 2006a). Overall, only really mild pre-rRNA processing defects, or no defects at all, were observed for large subunit pre-rRNA processing helicases, however all mutants studied had a growth defects. Comprehensive studies of the helicases of the small subunit revealed that over-expression of the dominant negative mutants resulted in accumulation of 23S, which suggests the inhibition of A2 cleavage, but no specific phenotype was observed (Granneman et al., 2006a).
DExH-box helicase Mtr4p was shown to play a role in 5.8S maturation and exosome activation (de la Cruz et al., 1998). Analysis of Mtr4p mutants revealed accumulation of extended 5.8S species (Allmang et al., 1999), which indicates that Mtr4p might play a role in remodelling of some secondary structures in ITS2.

As the question about the potential targets of DExD/H-box helicases in pre-rRNA processing remains open, current studies are concentrated on investigation of the interaction of helicases with pre-rRNA, which might help to identify their potential targets.

### 1.2.1 Introduction to splicing

Coding (exonic) sequences of most eukaryotic genes are interrupted by non-coding sequences (introns). At the time this discovery was made, the advantage in possessing interrupted genes was not really obvious. While analysing genomes it was noticed that many proteins are composed of a patchwork of active modules with borders of the domains corresponding to exon-introns boundaries. Therefore the presence of interrupted genes might be evolutionally advantageous as it allows increased genome capacity without increased size and combines existing active units to generate proteins with new functions.

Seven types of introns were identified so far, but only two of them were identified in eukaryotic protein coding genes (U2-dependent and U12-dependent). Analysis of intron distribution shows that generally, lower eukaryotes have fewer introns. Thus around 250 introns were identified in the genome of the yeast *Saccharomyces cerevisiae*, while individual human genes might contain more than 100 introns (collagen Type VI contains 117 introns). Although only approximately 4% of *Saccharomyces cerevisiae* genes contain introns, many intron-containing genes in yeast are highly expressed. For example, most of the ribosomal proteins genes contain intron, therefore intron-containing transcripts comprise approximately 25% of all transcripts in yeast (Ares et al., 1999; Lopez and Seraphin, 1999).
cerevisiae introns are mainly distributed towards the 5'of genes. The extremely 5'-biased intron distribution of genes in the yeast genome is likely driven by secondary intron loss via intronless cDNAs produced by reverse transcription of a processed mRNA (Fink, 1987).

The process of removing intronic regions from precursor mRNA (pre-mRNA) transcripts is called pre-mRNA splicing. Pre-mRNA splicing is catalysed by a large ribonucleoprotein complex called the spliceosome and research shows that all known spliceosomes rely on the same mechanism of splicing reaction (Brody and Abelson, 1985; Grabowski et al., 1985). Two distinctive classes of spliceosomes were described, which perform splicing on U2 or U12 introns. The canonical (U2-dependent) or major spliceosomes are universal, while minor spliceosomes (U12-dependent) are present only in metazoans and plants and are able to catalyse splicing of an atypical class of introns (Hall and Padgett, 1994, 1996).

1.2.2 Sequences important for splicing
Precise definition and excision of introns is a necessity in living cells. Incorrectly spliced RNA can potentially lead to reduced viability or death of the cell as in most cases incorrectly spliced pre-mRNA can cause premature stop codon inclusion and therefore promote RNA degradation through NMD. Recent advances in large-scale studies with human material revealed that incorrect splicing determines multiple diseases, or accounts for disease severity, therefore correct excision of the introns is extremely important. Correct choice of splice sites is defined, on one hand, by cis-elements in sequences in the RNA, and on the other hand, by a wide range of trans-acting factors of the spliceosomal machinery.

Three short conserved sequences were shown to be important for splicing in yeast: the 5' splice site, the 3'splice site and branch-point sequence.
Mutagenesis analysis identified the consensus sequence of the 5'splice site in yeast as AG/GUAUGU, with the first two nucleotides of intron being extremely conserved, and changes in these nucleotides were shown to abolish splicing completely (Vijayraghavan et al., 1986). Another important intronic sequence is usually located 20-60 nucleotides upstream of the 3'SS, and has a sequence UACUAAC. The last A of this sequence is a highly conserved adenosine residue, which provides the nucleophile for the first attack in first splicing reaction, and is the site where intron-lariat forms a branch (branch-point).

Introns in yeast and higher eukaryotes have the dinucleotide AG at their 3' end. In addition, 3'SS in mammals is preceded by a stretch of pyrimidine bases located just upstream. This stretch is called the polypyrimidine tract and it affects efficiency of 3'SS choice in alternative splicing. The polypyrimidine tract is rarely seen in yeast introns, and the 3'SS choice is determined mainly by the branch-point sequence.

Mutagenesis of the consensus sequences in pre-mRNA in yeast either abolishes splicing completely, or results in accumulation of aberrant intermediates. On the contrary, in mammals nucleotide changes in splice sites usually result in using cryptic splice sites, which probably reflects the difference in spliceosomal machineries between yeast and higher eukaryotes. In higher eukaryotes, multiple trans-acting factors affect splice site selection, in addition to cis-elements encoded in the RNA.

1.2.3 Pre-mRNA splicing reaction
Introns are removed from pre-mRNA by two sequential trans-esterification reactions. In the first step of the splicing reaction, the phosphodiester bond at the 5'SS is cleaved as a result of nucleophilic attack by the conserved adenosine (branch point nucleotide) located upstream of the 3'SS. This step generates a 5'exon intermediate exonI with a free 3'-hydroxyl group and a branched intron-exon II (branched intron...
is often referred to as lariat) with a 2'-5' bond at the branch site. In the second step, attack by the 3'hydroxyl of exon1 at the 3'SS results in joining of the two exons together via a 3'-5' phosphodiester bond. This produces two products of the splicing reaction: mRNA and excised intron-lariat (Ruby and Abelson, 1991).

**Figure 1.6 Schematic representation of pre-mRNA splicing reaction.** Splicing occurs via two trans-esterification reactions. Products and intermediates of the splicing reaction are shown.

### 1.2.4 The spliceosome

The spliceosome is a large and dynamic ribonucleoprotein complex, consisting of 5 small nuclear ribonucleoprotein particles (snRNPs) and non-snRNP proteins. Each snRNP contains an snRNA associated with the defined set of proteins (Guthrie and Patterson, 1988).
Spliceosomal snRNAs form a highly conserved and dynamic network of RNA-RNA interactions, which plays a primary role in recognition of splice sites (SS) and is likely to be the key elements in catalysis. Consistent with this, U1, U2, U4, U5 snRNAs have extremely conserved primary structure in functionally important regions, but vary in length and sequence among different species. At the same time U6 snRNA is highly conserved in sequence and structure, which might represent the preservation of the important function of U6 sRNA in pre-mRNA splicing.

Some of the snRNP proteins are the common components of several snRNPs, like Sm-proteins, while others are specific to individual snRNPs. Seven Sm-proteins designated B, D1, D2, D3, E, F, G are components of U1, U2, U4, U5 snRNPs, and recognize a highly conserved Sm-motif on snRNAs. U6 snRNP does not contain Sm-proteins, instead it is associated with Sm-like proteins called Lsm proteins for their similarity and possession of Sm-like domains.

Additionally, several spliceosomal proteins are not associated with snRNPs. These proteins are involved in stabilization of snRNP association with the spliceosome, play roles in rearrangements of RNA-RNA and RNA-protein interactions and spliceosome recycling.

1.2.5 Spliceosome assembly

Spliceosome assembly is a dynamic and ordered process. It involves recognition of splice sites on the pre-mRNA by snRNAs, and results in assembly of catalytically competent spliceosome able to perform the first step of splicing (Brow, 2002).

Spliceosome assembly is initiated by basepairing of the 5’ end region of U1 snRNA with a conserved complementary sequence at the 5’ end of the intron in the pre-mRNA in an ATP-independent manner (Ruby and Abelson, 1988). As a result commitment complex (CC) in yeast, or E’ complex in mammals is formed (Seraphin
and Rosbash, 1989). It was shown that recruitment of U1 snRNA to the nascent transcript might occur co-transcriptionally, however the role and significance of co-transcriptional splicing in yeast is debated. While it is accepted that spliceosome assembly might occur co-transcriptionally, the large proportion of splicing in yeast is believed to be transcription-independent.

On the next step base pairing between U2 snRNA and the branch point sequence occurs, forming Complex A in yeast. First, BBP (Branch point Binding Protein) - Mud2 heterodimer recognizes the branch-point sequence, and the KH domain of BBP specifically binds the branch-point sequence and flanking nucleotides (Abovich et al., 1994; Abovich and Rosbash, 1997). BBP-Mud2p dimer is believed to serve as a bridge between 5'SS and branch-point as interactions between U1 snRNP proteins Prp39p and Prp40p and BBP were reported (Abovich and Rosbash, 1997; Kao and Siliciano, 1996). Additionally, Mud2p interacts with Clf1p (Prp19 complex protein) and Sub2p suggesting that BBP-Mud2p dimer also serves as an anchor for the proteins involved in later stages in splicing (Abovich and Rosbash, 1997; Wang et al., 2005). Further U2 snRNP recruitment to CC2 complex occurs, and it is believed to be initially based on protein-protein interactions (Gozani et al., 1998). Before stable association of U2 snRNA with the branch-point occurs, U2 snRNA undergoes ATP-dependent structure rearrangements and BBP is removed from the branch-point allowing U2 snRNA to take its place (Zavanelli and Ares, 1991; Zavanelli et al., 1994). DexD/H box helicases Sub2p and Prp5p are implicated at this stage of spliceosome assembly (Dalbadie-McFarland and Abelson, 1990; Perriman and Ares, 2000). It was suggested that Prp5p alters the conformation of U2 in an ATP-dependent manner, and enables it to associate with the branch-point (Perriman et al., 2003). Study of Prp5p mutants with altered ATPase activity supported a role for Prp5p in stable association of U2 snRNA with the branch-point sequence (Xu et al., 2004; Xu and Query, 2007). While Prp5p function was characterised very well, the function of Sub2p in spliceosome assembly remains elusive. While depletion or inactivation of Sub2p affects spliceosome assembly, this protein was also shown to be involved in mRNA export in yeast (Jensen et al., 2001; Libri et al., 2001; Zhang and Green, 2001). On the contrary, the human homologue of Sub2p, protein-
hUAP56, is the only protein involved in stable U2 snRNA association with the spliceosome (Fleckner et al., 1997; Zhang and Green, 2001).

The mechanism of U4/U6.U5 tri-snRNP complex binding to Complex A is not well understood. However, certain evidence suggests that the U5 snRNP might target tri-snRNP to the spliceosome. Thus, the U5 snRNP component Prp8p was shown to interact with Prp39p and Prp40p in yeast two-hybrid screen (Abovich and Rosbash, 1997). Additionally, psoralen crosslinking of U1 and U5 snRNAs was observed, indicating that U5 snRNA might be the first component of the tri-snRNP to establish interaction with the Complex A (Newman et al., 1995; Wassarman and Steitz, 1992; Wyatt et al., 1992). This idea is supported by the fact that Aar2-associated U5 snRNP was consistently co-isolated with U1 snRNA (Gottschalk et al., 2001).

According to the currently accepted model, snRNPs assemble on pre-mRNA in a stepwise manner. Isolation of a penta-snRNP and tetra-snRNP challenged the stepwise assembly hypothesis (Stevens et al., 2002). It was argued that while penta-snRNP was isolated under very mild conditions similar to those in cells, other spliceosomal complexes were isolated under stringent conditions and likely to be artefacts of the isolation procedures (Stevens et al., 2002). This led to the proposition that the spliceosome does not assemble in an ordered manner, but rather interacts with pre-mRNA pre-assembled. However, lately several groups demonstrated co-transcriptional recruitment of U1 snRNA to pre-mRNA, which supports the stepwise assembly theory (Kotovic et al., 2003; Lacadie and Rosbash, 2005), and studying of arrested complexes in vivo favours step-wise spliceosome assembly (Tardiff and Rosbash, 2006).

1.2.6 Towards a catalytic spliceosome

After spliceosome assembly, a series of activation steps take place, which result in formation of a catalytically competent spliceosome. Rearrangements include dissociation of U1 and U4 snRNAs and assembly of U2/U6 duplex, which presumably comprises the catalytic centre of the spliceosome (Madhani and Guthrie, 1994; Staley and Guthrie, 1998; Valadkhan, 2005). Additionally, new interactions
between 5' exon and U5 snRNA, and 5' intron and U6 snRNA are formed (Sontheimer and Steitz, 1993).

To activate spliceosomes, first U1 snRNA must be displaced from the 5'SS, and U6 interaction with the 5'SS takes place. Before that, extensive base-pairing of U4/U6 duplex must be disrupted. It was noticed that U1 snRNA release requires unwinding of U4/U6 duplex, suggesting that these two events are likely coupled (Kuhn et al., 1999; Staley and Guthrie, 1999). Recent data indicated that a highly conserved InteRNAI Stem-Loop of U6 snRNA plays a role in regulation of the coupling of the release of U1 and U4 snRNAs from the spliceosome, possibly by stabilising the U6/5'SS interaction (McManus et al., 2007).

DEAD-box helicase Prp28p was shown to promote U1 snRNA release from the spliceosome, and Brr2p was implicated in U4/U6 unwinding (Raghunathan and Guthrie, 1998; Staley and Guthrie, 1999) as Brr2-1 mutant fails to release U4 snRNA from complex B. It was shown that Snu114p derepresses Brr2p during spliceosome activation. Also a mutation in the tri-snRNP protein, Prp38p, affects U4 release, suggesting that Prp38p affects Brr2 function (Xie et al., 1998). Strikingly, several alleles of Prp8p suppress brr2-1 and prp28-1 alleles suggesting that Prp8p might act as a switch, promoting unwinding of U4/U6 and release of U1 snRNA directly or indirectly (Kuhn et al., 1999; Kuhn et al., 2002).

Immediately after or concomitantly with U4 snRNA release, a protein complex consisting at least eight components (Prp19 complex; NTC) joins the spliceosome (Chen et al., 2002). It is believed that Prp19 complex stabilizes U6 and U5 snRNAs interactions with pre-mRNA after U1 and U4 release (Chan and Cheng, 2005; Chan et al., 2003). It was proposed that the presence of Prp19-mediates stabilization of U6 interaction with pre-mRNA through remodelling of U6 snRNP (Chan and Cheng, 2005). During the remodelling, Lsm proteins are removed from the 3'end of U6 allowing it to establish novel RNA-RNA interaction within the intron (Chan et al., 2003). Also it was proposed that U6/5'SS and U5/exon5' interactions are dynamic during tri-snRNP association with the spliceosome, and addition of the NTC
stabilizes or "locks" the interactions (Chan and Cheng, 2005). Thus initial U6 interaction with the intron involves two sets of conserved ACA triads. After the NTC joins the spliceosome switch of ACA 42-44 box to ACA 47-49 box occurs, generating active spliceosomes. Additionally, while immediately after tri-snRNP association U5 snRNA establishes dynamic interactions between U5 and pre-mRNA, in the presence of the NTC the U5 interaction with 5' exonic sequence becomes strictly defined (Chan and Cheng, 2005).

DEAH-box helicase Prp2p is required for the final step of spliceosome activation, however the nature of the activation events is not known (Kim and Lin, 1996). It was hypothesised that the spliceosome might change its conformation to gain catalytic competence, however nothing is known about the Prp2p-dependent rearrangements. Interestingly, dominant negative forms of Prp2p can be cross-linked to pre-mRNA, suggesting that pre-mRNA might be the target of Prp2p activity (Edwalds-Gilbert et al., 2004; Teigelkamp et al., 1994).

Recently obtained data exposed a potential role of U2 snRNA in regulation of spliceosome conformations. It was noticed that U2 snRNAs fluctuates between two competing conformations, which are compatible with different stages of splicing (Hilliker et al., 2007; Perriman and Ares, 2007). These conformations, stem-loop IIα and stem-loop IIc, are mutually exclusive forms of U2 snRNA (Hilliker et al., 2007; Perriman and Ares, 2007). Numerous studies showed that stem-loop IIα is necessary for spliceosome assembly, while mutations hyperstabilizing stem-loop IIc inhibit pre-spliceosome formation (Zavanelli et al., 1994). Rearrangement of U2 snRNA between steps will be discussed in detail in the next section.
1.2.7 The catalytic spliceosome.

After U4 release, U6 snRNA forms a duplex with U2, however this event is not well understood. Several lines of evidence suggest that U6 structure undergoes rearrangements, suggesting the existence of some intermediates of U2/U6 duplex (Brow and Vidaver, 1995). On the contrary UV cross-linking suggests that U2/U6 duplex starts forming even before U4/U6 duplex is unwound (Frilander and Steitz, 2001).

The U2/U6 duplex composes the catalytic centre of the spliceosome (helix II). The U2/U6 snRNA duplex shares structural similarity and demonstrates interchangeability of domains between U2/U6 duplex and group II introns, suggesting that the spliceosome is likely a ribozyme (Hetzer et al., 1997; Shukla and Padgett, 2002). Supporting this notion, recently the ability of fragments of U2/U6 to catalyze a first-step like reaction was demonstrated in a cell-free system (Valadkhan and Manley, 2001). Strikingly, the loop region of U5 snRNA can be dispensable for this reaction. As the invariant loop of U5 snRNA interacts with both exonic sequences it is proposed that U5 snRNA aligns the exons during the splicing reaction for ligation (Newman and Norman, 1992; O'Keefe et al., 1996; Sontheimer and Steitz, 1993; Wyatt et al., 1992), and this explains how the first-step like reaction might occur in the absence of U5. While interaction of U5 with the 5’ exon is formed during spliceosome activation, interaction of U5 loop with the 3’exon occurs only after the first step is complete (Cortes et al., 1993; Newman and Norman, 1991; Newman and Norman, 1992; Newman et al., 1995). It is not entirely clear how the U5 loop interacts with exonic sequences, however the protein “in the heart of the spliceosome” Prp8p is proposed to serve as a scaffold for U5 snRNA/substrate interaction, and substrate repositioning during catalytic steps (Grainger and Beggs, 2005). It was noticed that certain mutant alleles of Prp8 inhibit one step of the splicing while promoting another one (Konarska et al., 2006; Liu et al., 2007).

The catalytic centre is not rigid, and undergoes changes, as the second step of splicing is not simply the reverse reaction of step one (Burgess and Guthrie, 1993; Konarska et al., 2006). Thus, the branch-point should be removed from the catalytic
centre and substituted with the 3’SS. Supporting this reorganization, products of rare trans-splicing events were discovered (Smith et al., 2007). Further, it was discovered that sequences of U2 snRNA located downstream of branch-point interaction region fluctuate between 2 mutually exclusive structures (Stem IIa and Stem IIc) during spliceosome assembly and trans-esterification reactions. While Stem IIa promotes toward the catalytic spliceosome, Stem IIc is necessary for the first catalytic reaction (and likely for the second one), and is restored back to Stem IIa after the step 1 reaction is completed (Hilliker et al., 2007; Perriman and Ares, 2007). Toggling of U2 structure supports the idea that binding of U2 to the branch point helix could be disrupted during splicing as Stem IIc prevents U2 binding to the branch-point, and supports the idea of a flexible catalytic centre.

In between catalytic steps not only RNA-RNA rearrangements occur, but several second step specific factors (Prp17p, Slu7p) were shown to join the spliceosome (Jones et al., 1995). These factors include DEAH-box helicase Prp22p, which is required for the second trans-esterification reaction, and ATP-dependent release of mature mRNA from the spliceosome (McPheeters et al., 2000).

1.2.8 The post-spliceosome.

After splicing occurs, the products of the splicing reaction (mRNA and excised intron-lariat) must be released and U2/U6.U5 snRNPs dissociate. DEAH box protein Prp22p was proposed to unwind mRNA from the loop of U5 snRNA, thus promoting mRNA release (Schwer, 2008). However, much less is known about events, which take place after mRNA release. No post-splicing complex(es) were isolated so far, and no models of spliceosome disassembly were proposed. However, two possibilities of the mode of spliceosome disassembly exist. First, by analogy with the step - wise spliceosome assembly process, spliceosome disassembly might require several stages. Alternatively, after mRNA release post-splicing complex might fall apart releasing individual snRNPs and excised intron. Before snRNPs get released, several RNA-RNA rearrangements take place. Thus in the post-splicing complex, U2 still remains base-paired with U6, and this duplex must be disrupted in order for U2
and U6 to undergo recycling. Additionally, U2 snRNA is still associated with the excised intron lariat. At the same time, after mRNA leaves the post-splicing complex, no known RNA-RNA interactions between U5 snRNA and remaining snRNAs and excised intron-lariat exist, and it is not known when U5 leaves the spliceosome.

Several factors were shown to play a role in intron-lariat release/spliceosome disassembly. Initially, the ATPase activity of DEAH-box helicase Prp43p was shown to be required for lariat release as ATPase deficient mutants of Prp43p accumulate excised intron in the post-splicing complex (Martin et al., 2002). Recently mutants of several other splicing factors were found to affect lariat release. Thus, mutant of DExH box helicase Brr2p was found to accumulate excised intron-lariat (Small et al., 2006). Interestingly, U2/U6 duplex was found to stimulate helicase activity of Brr2p in vitro (Xu et al., 1996), suggesting that Brr2p unwinds U2/U6 in addition to its function in unwinding U4/U6 duplex, providing an example of a protein playing opposite functions during the splicing reaction. The activity of Brr2p in spliceosome disassembly, as in spliceosome assembly, is governed by Snu114p and Prp8p (Small et al., 2006). The fact that proteins involved in spliceosome disassembly have function elsewhere in the spliceosome (Brr2p, Snu114p, Prp8p) or in different RNA processing machinery (Prp43p) explains the lack of information about spliceosome disassembly and difficulties of isolation of the post-splicing complex.

1.2.9 DExD/H box proteins as fidelity factors.

All biological systems rely on programmed intrinsic ability to proofread the transfer of the information, and discard aberrant molecules/intermediates, which are below a certain standard. Moreover, most of the reactions in multi-step biological processes are bi-directional, therefore in order to complete the cycle or reaction, some kind of driving force is often necessary. The driving force provides directionality to the biological reactions by introducing irreversible steps into the pathways; and one of the commonly used irreversible reactions is ATP hydrolysis. Combining two necessities for the cell, it was postulated that not only the fact of the hydrolysis, but also the rate of ATP hydrolysis is important, as it was suggested that rate of ATP
hydrolysis serves as a proofreading mechanism by providing a limited amount of
time for the reaction to occur.

It was noticed in biochemical analysis and by observation of the splicing of the group
II of introns that the net change of energy during a splicing reaction is zero, which
indicates that the splicing reaction per se does not require ATP hydrolysis and
possibly can occur spontaneously (Konarska and Query, 2005). However, ATP was
shown to be utilized by multiple DExD/H box helicases during the splicing process,
and therefore a role for DExD/H box helicases as driving force and fidelity factors
was proposed.

In a pioneering screen for factors that reduce splicing fidelity, the DEAH box
helicase Prp16p was identified (Burgess et al., 1990). It was observed that
compromising Prp16p ATPase activity causes suppression of branch-point mutants,
and therefore allows aberrant splicing to take place. This observation led to the
introduction of a kinetic proofreading theory as a mechanism of fidelity in splicing
(Staley and Guthrie, 1998). The main postulate of this hypothesis is that ATP
hydrolysis determines rearrangement of the catalytic centre independently of the
splicing reaction. It is accepted that "correct" or "right" substrate will have the best
conformation/sequence to base-pair with snRNAs, while the absence of
complementarity of aberrant substrate causes delay in base pairing. If the act of ATP
hydrolysis by DExD/H box helicase occurs after the right conformation was
achieved, the substrate goes to the next level of processing, if before – substrate is
not processed, and gets discarded.

After discovery of the role of Prp16p as a fidelity factor, several other splicing
helicases were found to employ similar mechanism for proofreading. Alterations of
Prp22p and Prp5p activities were shown to affect 3'SS and branch-point/U2
interactions respectively (Mayas et al., 2006; Xu and Query, 2007). In mRNA
surveillance, Upf1p, an RNA helicase of SF1 superfamily, was suggested to use ATP
hydrolysis to distinguish proper translation termination events, suggesting that
kinetic proofreading might be used in many aspects of RNA metabolism (Hilleren and Parker, 1999). It is possible that DExD/H box helicases in pre-rRNA processing play a role of fidelity factors in addition to their role in complex remodelling as suggested by kinetic proofreading.

1.3.1 Ribosomal biosynthesis.
The yeast ribosome consists of 4 different types of rRNAs and associated proteins. 18S RNA and associated proteins together create the small subunit (40S), and 25S, 5.8S and 5S with respective proteins compose the large (60S) subunit. The process of ribosome biogenesis is initiated with transcription of pre-rRNA precursors, and concomitantly with the transcription, processing steps and assembly of ribosomal proteins on the pre-rRNA precursors starts. Following initial assembly steps, pre-ribosomes are transported to the cytoplasm, where the final maturation steps occur (reviewed in (Henras et al., 2008; Venema and Tollervey, 1999). Most of the events of pre-rRNA processing take place in a specialised part of the nucleus called the nucleolus, although late steps of 60S maturation take place in the nucleus and late 40S maturation occurs in the cytoplasm.

25S, 18S and 5.8S are transcribed as a single precursor (35S), which undergoes a series of nucleolytic cleavages and extensive covalent modifications in order to produce mature rRNA species. In the 35S RNA, the sequences of the 18S, 5.8S and 25S species are separated by interRNAI transcribed spacers (ITS1 and ITS2), and flanked by exteRNAI spacers (ETS1 and ETS2) as presented in figure 1.7.
Figure 1.7 A. Organization of 35S operon of *S. cerevisiae*: The organization of 35S pre-rRNA precursor and location of processing sites. B. The pre-rRNA processing pathway in *S. cerevisiae*. The 35S precursor is generated by co-transcriptional cleavage at site B₀ within the 3ETS. This is followed by endonucleolytic cleavages at sites A₀, within 5'ETS, A₁ at the 5'end of the mature 18S rRNA and A₂ within ITS₁. Cleavage at A₂ separates the precursors to the 40S and 60S subunits and generates the 20S and 27SA₂ pre-rRNAs. 20S is subsequently exported from the nucleus to the cytoplasm where maturation to 18S is completed. The 27SA₂ pre-rRNA follows one of two alternate pathways: approximately 85% is cleaved at A₃ site within ITS₂, followed by 5'→3' exonucleolytic processing generating the 27SB₁₅ pre-rRNA. The remained 15% is processed at site B₁₁, yielding the 27SB₁₁ pre-rRNA. These two alternate forms of 27SB are cleaved within ITS₂ at site C₂, yielding 26S and 7S. The 7S is converted to 5.8S by a multistep 3'exonuclease pathway. Maturation of 26S to 25S rRNA similarly proceeds by a two-step 5' exonuclease pathway. (Adapted from E.Thomson and D.Tollervey, 2005)
90S particles

Ribosome biogenesis starts with the co-transcriptional cleavage of the nascent transcript at the B0 site in 3'ETS by Rnt1p, which generates a 90S particle containing 35S pre-rRNA (Kufel et al., 1999). Notably, mass-spectrometry studies of the 90S particle revealed that it is largely composed from U3 snoRNP, components required for small subunit processing, and also includes r-proteins of the small subunit (Grandi et al., 2002). It was shown that association of ribosomal proteins might play a role in pre-rRNA processing events, possibly by providing a scaffold for binding of pre-rRNA processing factors (Ferreira-Cerca et al., 2005). It was suggested that pre-60S particles assemble on pre-rRNA after the small and large subunit processing pathways are separated (Lebaron et al., 2005), however recent findings were reported of some of the pre-60S proteins with 90S particles, indicating that at least several pre-60S might be a part of the 90S particle (Oeffinger et al., 2007). Analysis of the protein content of 90S suggested the directionality of the assembly of processing factors and pre-rRNA proteins as 35S transcript is synthesised, supporting the idea that 90S assembly occurs co-transcriptionally (Fatica and Tollervey, 2002).

It is believed that the first set of proteins to become associated with 35S belongs to t-UTP (U Three Protein) complex, which is required for optimal transcription of pre-rRNA as well as early cleavages (Gallagher et al., 2004). Following association of t-UTPs with 35S, U3 snoRNP and UTPB and UTPC complexes join 35S (Gallagher et al., 2004). U3 snoRNP establishes direct interaction with pre-rRNA, and is required for early cleavages (Hughes and Ares, 1991). Additionally U3 snoRNA possibly acts as a chaperone preventing premature central pseudoknot formation in 18S sequence when it is still part of 35S (Beltrame and Tollervey, 1995; Sharma and Tollervey, 1999). It was suggested that the U3 snoRNA associated DEAH-box helicase Dhr1p plays a role in remodelling/annealing of U3 to the 18S sequence during pseudoknot formation, however the precise function of Dhr1p is not known (Colley et al., 2000). After extensive modification, 35S is cleaved in ETS1 at the A0 site, producing 33S species, which is subsequently cleaved at the A1 site, generating 32S pre-rRNA. 32S species are rapidly cleaved at A2 site, producing 20S and 27SA2, which are
precursors for the small and large subunit processing pathways. Enzymes responsible for these cleavages remain elusive, but there is evidence that these cleavages occur endo-nucleolytically. Mutations or defects that affect cleavages at A₀, A₁, or A₂ sites do not inhibit cleavage at A₃ site, which results in production of 23S/22S/21S and 27Sₐ₃ pre-rRNAs. It is believed that 23S pre-rRNA is an unsuitable substrate for 18S biosynthesis, as its accumulation usually correlates with the defect of 18S production (Allmang et al., 2000). Surprisingly, 21S precursor is converted to 18S, suggesting that bypassing cleavage at A₂ site does not always result in aberrant small subunit processing. 27Sₐ₃ precursor, on the other hand, is a normal substrate for the large subunit processing pathway (Vos et al., 2004).

Cleavage at the A₂ site separates two processing pathways, which lead to production of mature rRNAs, and it promotes dissociation of 90S particle into pre-40S and pre-60S.

**Small subunit biosynthesis**

As a result of the cleavage at A₂ 20S pre-rRNA is formed, which is rapidly exported from the nucleolus to cytoplasm, where the final step of 18S maturation takes place (Udem and Warner, 1973). 20S is converted to 18S via cleavage at site D located at the 3' end of mature 18S. It is believed that putative endonuclease Nob1p is responsible for the cleavage at site D (Fatica et al., 2003). Nob1p has a putative RNA binding domain, and a PIN-domain, a conserved motif found in several endonucleases that was proposed to determine RNase activity (Fatica et al., 2004). *In vivo*, depletion of Nob1p severely inhibits conversion of 20S to 18S in the cytoplasm. Interestingly, determinants of the cleavage at D site are thought to be sequence-specific, as nucleotide substitution was shown to abolish the cleavage completely. It is believed that premature cleavage might be prevented by secondary structure of site D, which is likely to be remodelled before the cleavage takes place (Fatica et al., 2003).

Following cleavage at A₂, pre-40 particle is released from 90S. This particle contains a few non-ribosomal proteins, and during 40S maturation only several protein factors
appear to join the pre-40S particles (Schafer et al., 2003). These mostly required for pre-40S export through the nuclear pore.

**Large subunit biosynthesis**

In comparison to small subunit biosynthesis, processing of 25S and 5.8S involves endonucleolytic cleavages and exonucleolytic digestion, and processing events can go two alternative ways (major and minor), which results in production of 25S and either short 5.8SS or long 5.8SL species. The functional difference between long and short forms of 5.8S RNAs is unknown.

Correlating with the complexity of the processing events, several pre-60S complexes were identified. These complexes correspond to different stages of pre-60S RNA processing, and they defined by the association of certain non-ribosomal protein factors with characterised role in 60S assembly (Fatica et al., 2002; Saveanu et al., 2001; Saveanu et al., 2003). Therefore analysis of protein content of pre-60S particles shows that early pre-60S particles are associated with numerous processing factors, however the number of the associated proteins appear to decline as maturation proceeds and particles are transported from the nucleolus to the nucleoplasm, and subsequently to the cytoplasm (Nissan et al., 2002).

Processing events leading to production of large subunit rRNAs are initiated by the cleavage at of 27SA2 at A3 site, which is performed by RNAse MRP (Henry et al., 1994; Schmitt and Clayton, 1993). RNAse MRP is a ribonucleoprotein complex, and consists of catalytic RNA and nine proteins; all the components were shown to be essential for the function of RNAse MRP (Chamberlain et al., 1998). Following cleavage at the A3 site, the end of 5.8S RNA is formed as a result of 5'-3' exonucleolytic activity of nucleases Rat1p and Xrn1p (Henry et al., 1994). Exonucleolytic digestion stops at site B1s, and results in formation of 5.8SS species (Henry et al., 1994). In the minor pathway, 27SA3 is cleaved at B1L, which produces the 5’end of 5.8SL (Faber et al., 2006). Following their formation, 27SB1s and
27SB₁₁ undergo the same processing events, which results in removal of ITS2, and formation of 5.8S and 25S species. Endonucleolytic cleavage at the C₂ site of 27SB by an unidentified enzyme generates 7S and 26S precursors. In a final step mature 25S is produced through trimming of 5' of 26S by Rat1p and Xrn1p (Geerlings et al., 2000). At the same time, maturation of 7S to 5.8S is likely to be a multi-step process, involving several exonucleases, including the exosome (Mitchell et al., 1997; Mitchell et al., 1996). DExH-box helicase Mtr4p is believed to couple rearrangements of highly structured ITS2 region with assisting exosome during digestion (Allmang et al., 1999; de la Cruz et al., 1998), followed by further nucleolytic digestion, resulting in formation of 5.8S species.

1.4 Crosstalk
Ribosome biogenesis is an extremely important process in living cells. It involves coordinated transcription by all three RNA polymerases, which produce pre-rRNA precursors and pre-mRNA of ribosomal proteins and pre-rRNA processing factors. Pre-rRNA transcripts undergo a series of processing events and assemble with ribosomal proteins and pre-rRNA processing factors. Obviously, this complicated process requires to be concerted and regulated. It was shown that under stress conditions transcription of pre-rRNA is likely to be affected as a result of action of the TOR network (Mayer and Grummt, 2006). In support of this, it was noticed that all ribosomal proteins create a regulon and therefore are likely to be co-regulated at the transcription level (Wade et al., 2006). Specific promoter elements of the ribosomal genes are likely to play a role in this process.

Recently more and more pre-RNA processing factors were found to be shared subunits between different processes. Thus links between pre-rRNA processing and the cell cycle (Dez and Tollervey, 2004), and between pre-rRNA and splicing were identified (Lebaron et al., 2005). Links between pre-rRNA processing and splicing is an object of interest of this thesis, and will be discussed in detail.
So far, several splicing factors were implicated in pre-rRNA processing. One of them, Snu13p, was found to be a component of both U4/U6.U5 tri-snRNP and C/D box snoRNPs (Watkins et al., 2000). It was established that Snu13p binds directly to U4 snRNA, and is required for spliceosome assembly (Nottrott et al., 1999). Experiments with the human orthologue of Snu13p, protein 15.5K, revealed that 15.5K binds to the C/D box motif (Vidovic et al., 2000). Interestingly, the C/D box motif has a striking structural resemblance to the binding site of Snu13p in U4 snRNA, and mutagenesis analysis suggests that RNA secondary structure rather than sequence is required for 15.5K binding to RNA (Watkins et al., 2000). Despite U4 snRNP and C/D box snoRNP having very distinctive functions, a number of similarities can be drawn. In addition to structural similarities of RNA-15.5 interactions, there is an unusual analogy in the protein content of the RNPs. U3 snoRNP proteins Nop56p and Nop58p which have a conserved NOP domain, and this was also identified in U4 snRNA component Prp31p (Watkins et al., 2002). Also functional similarity between these RNPs can be identified as both classes of RNPs play roles as chaperons in RNA processing. Taken together these similarities are likely to indicate a common origin of U4 snRNP and C/D snoRNPs (Watkins et al., 2000).

DExD/H-box protein Prp43p is a helicase shared between splicing and pre-rRNA processing (Combs et al., 2006; Lebaron et al., 2005; Leeds et al., 2006). While in splicing it was shown to participate in spliceosome disassembly, its function in pre-rRNA processing has not been identified yet. Prp43p was found to be associated with multiple pre-rRNA precursors of both small and large subunits, including 20S and mature 18S species. As conversion of 20S to 18S takes place in the cytoplasm, it suggests that Prp43p might be exported to the cytoplasm with pre-40S particles. In pre-rRNA processing, Prp43p was co-isolated with G-patch containing protein Pfa1p, which is a likely co-factor for Prp43p. Analysis of pre-RNAs associated with Pfa1p suggests that Pfa1p is a novel component of pre-40S particles (Lebaron et al., 2005). Additionally, Pfa1p localises to the nucleus and cytoplasm (where the late stages of 40S maturation take place) (Huh et al., 2003), which confirms that Prp43p is a component of the late pre-40S particles and might be associated with pre-40S in
the cytoplasm. Remarkably, Pfa1p was found to have genetic interaction with the splicing factor Spp382p, suggesting the existence of a cross-network link between splicing and pre-rRNA processing (Pandit et al., 2006). While such a link between processing machineries can be hypothesised as a connection or feedback mechanism, no direct evidence of this is known, and therefore it requires further investigation.
Aims of the project

The aims of the project were to characterise the newly identified splicing factors Spp382p and Ntr2p, and investigate their role as co-factors of DEAH-box helicase Prp43p in splicing and pre-rRNA processing.
Materials and methods

2.1.1 Enzymes and chemicals
Enzymes used in this study were purchased from NewEngland Biolabs, Promega, Roche and Startagene.
Standard laboratory reagents were purchased from Sigma, Amersham, Formedium, Difco, Fluka and Gibco.

2.1.2 Commonly used buffers

<table>
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<th>Buffer</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
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<td>AGK buffer (0.5l)</td>
<td>10mM Hepes (pH 7.9), 1.5mM MgCl₂, 50ml 200mM KCl, 10% Glycerol, water</td>
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<tr>
<td>Buffer B</td>
<td>0.1M Potassium Phosphate, pH 7.5, 1.2M Sorbitol</td>
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<tr>
<td>Dialysis buffer (2l)</td>
<td>20mM Hepes (pH 7.9), 100mM KCl, 0.2mM EDTA, 20% glycerol, 1mM DTT, 1 pellet of complete protease inhibitor/2l of dialysis buffer</td>
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<tr>
<td>Lysis buffer</td>
<td>100mM NaCl, 50mM Tris-HCl (pH 7.5), 0.1% TritonX-100</td>
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<tr>
<td>BPTE</td>
<td>12mM Pipes, 30.6mM Bis-Tris, 1.2mM EDTA</td>
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<td>Church buffer (mod)</td>
<td>0.25M Sodium Phosphate (pH 7.2), 7% SDS, 1mM</td>
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<td>2xIPP</td>
<td>12mM Hepes (pH 7.9), 0.3M NaCl, 5mM MgCl₂, 0.1% (v/v) NonindetP40</td>
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<tr>
<td>NTN</td>
<td>150mM NaCl, 50mM Tris-HCl (pH 7.5), 0.1% (v/v) NonindetP40</td>
</tr>
<tr>
<td>1xPBS</td>
<td>137mM NaCl, 2.7mMKCl, 3.3mM Na₂HPO₄, pH adjusted to 7.4</td>
</tr>
<tr>
<td>PBST</td>
<td>137mM NaCl, 2.7mMKCl, 3.3mM Na₂HPO₄, pH adjusted to 7.4, 0.1%(v/v) Tween-20</td>
</tr>
<tr>
<td>20xSSC</td>
<td>3M NaCl, 0.3M sodium citrate, pH 7</td>
</tr>
<tr>
<td>50xTAE</td>
<td>2M Tris, 50mM EDTA, 5.7% (v/v) acetic acid</td>
</tr>
</tbody>
</table>

2.2 Culture and media
E.coli strains were grown in Luria-Bertani broth (LB medium). If required Ampicillin was added prior use (100µg/ml).

LB medium
For 11 of medium
10g Bacto-tryptone
5g Yeast extract
10g NaCl
pH was adjusted to 7.2
For solid media 20g of agar was added to LB broth before autoclaving

Standard growth and handling techniques were used for propagation of *S. cerevisiae*. Yeast were grown in either rich (YPDA, YPGR, YPGRS) or minimal SD or SG media.

For 1 litre of the medium following components were mixed, dissolved in water and autoclaved. For solid media, agar in final concentration 2% (w/v) was added prior to autoclaving.

**YPDA:** 10g Yeast extract, 20g Peptone, 20g Glucose, adenine sulphate
**YPGR:** 10g Yeast extract, 20g Peptone, 20g Galactose, 20g Raffinose, adenine sulphate
**YPGRS:** 10g Yeast extract, 20g Peptone, 5g Galactose, 20g Raffinose, 20g Saccharose, adenine sulphate
**SD:** 1.7g Yeast nitrogen base, 5g Ammonium sulphate, 20g Glucose, amino acids as required
**SG:** 1.7g Yeast nitrogen base, 5g Ammonium sulphate, 20g Galactose, 20g Raffinose, amino acids as required

### 2.3 Bacterial strains
DH5α strain was used for propagation of all the plasmids.
BL21 strain was used for protein expression.

<table>
<thead>
<tr>
<th>Table 2.3 Bacterial strains</th>
</tr>
</thead>
</table>

<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<td>DH5α</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; gyrA96 (Nal&lt;sup&gt;r&lt;/sup&gt;) recA1 relA1 endA1 thi-1 hsdR17 glnV44 deoRA(lacZYA-argF) U169[Φ80dΔ(lacZ)M15]</td>
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<td>BL21</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, ompT, hsdS&lt;sub&gt;B&lt;/sub&gt;, (r&lt;sub&gt;B&lt;/sub&gt;-,m&lt;sub&gt;B&lt;/sub&gt;-), gal, dsm (DE3)</td>
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### 2.4 Yeast strains

Table 2.4 Yeast strains

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<th>Genotype</th>
<th>Source</th>
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<tr>
<td>BY4741</td>
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<td>Brachman et al., 1998</td>
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<td>BK4H</td>
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### 2.5 Plasmids

Table 2.5 Plasmids

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<td>pBS1479</td>
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<td>p113</td>
<td>( CEN, \text{PRP43} )</td>
<td>Martin et al., 2002</td>
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<td>pRS313</td>
<td>( CEN, \text{URA3} )</td>
<td>Sikorski and Hieter, 1989</td>
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<td>pTAG1</td>
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<td>This study</td>
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<td>pPRP43wt</td>
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<td>pPRP43E716A</td>
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2.6 Oligonucleotides

Oligonucleotides used in this study were purchased from Invitrogen.

Table 2.6 Oligonucleotides

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</tbody>
</table>

2.7 Antiseras

Table 2.7 Antiseras
2.8 Bacterial techniques
Growth, preparation of competent *E. coli*, and transformation of *E. coli* were followed as in Current Protocols in Molecular Biology (2001).

2.9 Yeast transformation
Transformation of yeast is based on high-efficiency lithium acetate method (Gleitz and Woods, 2002).

For yeast transformation overnight culture was diluted in fresh medium thus cells will undergo 2-3 divisions and culture still will be in logarithmic phase.

Culture (50ml) was harvested by centrifugation, washed 2 times with water, and pelleted. Cells were resuspended in transformation mix and placed on water bath for 45min at 42°C. After the heat shock samples were spun, supernatant was gently removed, resuspended in water and plated on selective media.

Transformation mix
240μl of PEG_{3000}
36μl of 1M LiAc
5-25μl of DNA (depends on whether plasmid or PCR product was used)
ssDNA -100μg
Water —to final volume 360μl

2.10 Recombinant DNA techniques
Standard DNA manipulations were carried out as described in Sambrook and Russel, 2001, or manufacturer’s instructions. These include agarose gel electrophoresis, ethidium bromide staining, phenol-chloroform extraction of nucleic acids, isolation of DNA fragments from agarose gels, restriction enzymes digests, dephosphorylation of DNA and ligation of DNA fragments.
2.11 Polymerase Chain Reaction

PCR was used for amplification of regions of yeast genome, identification of recombinant clones and generation of short substrate for in vitro splicing.

For yeast-colony PCR reaction using Taq polymerase (Roche) was used.

For cloning and substrate generation either Pfu (Promega) or Phusion (Finnzymes) polymerases were used.

The choice of the enzyme dictated the protocol used, and the reactions were assembled according to the manufacturer's instruction.

2.12 RNA techniques

2.12.1 RNA extraction

Approximately 2-5 OD600 of exponentially growing cells were harvested by centrifugation, washed with sterile water and pelleted. Pellets were snap-frozen and stored at -80°C.

100μl of Zirconia beads and 100μl of GTC/Phenol mix were added to each pellet. Samples were vortexed for 5 min at full speed at 4°C. After quick spin to remove liquid from the cap, 700μl of GTC/Phenol mix was added to the samples, which were subsequently incubated on a shaker at 65°C for 5 min. 150μl of 3M Sodium Acetate pH 5.2 and 350μl of Chloroform were added to the mix, vortexed, and spun for 10 min at 14000 rpm. The aqueous phase was removed and combined with an equal volume of Phenol/Chloroform/IAA. Samples were vortexed and spun as described above. Finally, 1ml of 100% Ethanol was added to aqueous phase, and samples were placed for 30 min - 2 hours at-20°C. RNA was collected by centrifugation for 30 min at 14000rpm (4°C). The pellets were washed with 70% ethanol, dried and resuspended in sterile water. Samples were stored at -80°C.

GTC mix
4M Guanidine Thicyanate
0.05M Tris pH 8
0.01M EDTA pH8
2% Sarcosyl
1% β-mercaptoethanol)
2.12.2 In vitro transcription
To prepare template for in vitro splicing reaction, plasmid p283 with shortened ACT1 gene under T7 promoter was used. Plasmid preparation (using Qiagen miniprep kit is essential as preparations obtained with other brands were contaminated with RNAseA) was either digested with BamHI enzyme, or used as a template for PCR to generate an aberrant RNA substrate. Digestion products or PCR were purified with PCR purification kit (Amersham), and used for in vitro transcription.

All the reaction components were added in the following order:

1) Template DNA, corresponding 0.01 ug/μl
2) rNTP mix, containing 5mM of rGTP, rCTP , rUTP and 0.2mM rATP 2μl
3) 100mM DTT (dithiothreitol) 2μl
4) 10x T7 transcription buffer 2μl
5) 10mM cap analog structure 1μl
6) Distilled water

mix to 20 μl of the final

7) 10mCi/ml (α-32P) rATP 2μl
8) 10 units of rRNAsin (Promega) 1μl
9) T7 RNA polymerase

The components were mixed by pipetting and incubated for 30 minutes (or slightly longer) at 37°C. Transcription reaction was terminated by placing the reaction mix on ice, then P/C/I purified, and ethanol precipitated. The pellet was resuspended in water, and mixed with 2x RNA loading buffer. Sample was run on 6% denaturing polyacrylamide gel, the transcript was visualised with autoradiography, and cut out of the gel. The gel slice was stored at -20°C until required. To elute RNA, gel slice was defrosted, mashed in 1.5 ml eppendorf tube using a tip, and resuspended in Elution buffer. Elution was performed at 37°C for 6 hours on shaking platform (Eppendorf). After the elution, supeRNAtant containing RNA transcript was
carefully removed (special care should be taken to avoid small pieces of the gel), and precipitated with Ethanol. After precipitation, pellet was resuspended in de-ionized water and stored at -20°C.

**Elution buffer 5x**
- 50mM Tris, pH 7.6
- 5% SDS
- 50mM EDTA pH 8.0

### 2.12.3 In vitro splicing

For *in vitro* splicing reaction the following components were mixed:

1. 0.6M Potassium phosphate buffer 1μl
2. 25mM MgCl₂ 1μl
3. 20mM ATP 1μl
4. 30%(w/v) PEG₈₀₀₀ 1μl
5. Splicing extract 4μl
6. Radiolabelled RNA
7. Distilled water to 10μl

The reaction mixture was incubated at RT for 25 min and terminated by putting the tube on ice. 3μl of Proteinase K solution (1mg/ml ProteinaseK 50mM EDTA, 1%(w/v) SDS) was added to the reaction, which was subsequently incubated for 45 min at 37°C. 200μl of splicing cocktail (50mM NaOAc, 1mM EDTA, 0.1% (w/v) SDS, 25μg of *E.coli* tRNA) was added to the samples, which were P/C/I extracted and ethanol precipitated. The samples were dried at RT from residual ethanol, resuspended in water, and mixed with an equal volume of the 2X RNA loading buffer (100% Formamide, a little bit of Bromphenol Blue and Cyanol Blue). Prepared samples were heated at 90°C for 3 minutes, placed on ice to cool down and loaded on 7% (w/v) denaturing polyacrylamide gel.
For immuno-precipitation of the products of *in vitro* splicing reaction 50-100μl splicing reactions were assembled, incubated for 30-40 min at RT, and mixed with an appropriate volume of 2xIPP buffer.

### 2.12.4 Co-immunoprecipitation of snRNAs/spliceosome.

35 μl of pre-hydrated PAS (Protein A sepharose) beads were washed 3 times with 1000μl of NTN buffer, and mixed with 300μl of NTN buffer, an appropriate amount of antibody and 5μl of Blocking buffer. Beads were incubated on rotating wheel for 1 hour at 4°C, washed twice in NTN buffer, and once with 1xIPP. After that beads were mixed with 50μl of 2xIPP, 0.5μl of RNAsin and 50μl of splicing extract or splicing reaction unless is stated otherwise.

**NTN buffer**

150mm NaCl

50mM TrisCl pH7.5

0.1%(v/v) Nonindet P40

**1xIPP**

6mM Hepes pH7.9

150mM NaCl

2.5mM MgCl₂

0.05%(v/v) Nonindet P40

**2xIPP**

12mM Hepes pH7.9

300mM NaCl

5mM MgCl₂

0.1%(v/v) Nonindet P40

**Blocking buffer:**

Final concentrations are:

100μg/ml of glycogen,

100μg of BSA,

100μg of tRNA

All the ingredients were mixed in NTN buffer, and stored at -20°C.
Immunoprecipitation was performed for 1 hour at 4°C on rotating wheel. After the precipitation beads were washed 3 times with NTN, and mixed with 200μl of Extraction buffer. For total samples 180μl of Extraction buffer were mixed with 20% of unbound supernatant; samples were processed as IPs described in 2.12.4. Obtained RNA samples were loaded on 6% polyacrylamide gel, and transferred to Hybond-N membrane. For northern blot the membrane was directly put into 50ml of hybridisation buffer (modified Church Buffer) with added labelled oligo(s) for at least 3 hours at 37°C, or overnight. Membrane was washed 2-3 times with 1xSSC at 37°C for 5-20 minutes, wrapped in Saran Wrap and exposed to MS film (Kodak).

**RNA extraction buffer**

50mM Tris-HCL, pH 7.5, 5mM EDTA, 1.4 (w/v) SDS,

End-labeling of the oligonucleotides for northern blot was performed in 20μl reaction for 1 hour at 37°C. Labelled oligonucleotides were purified through the 0.2μm filter to remove unincorporated radioactive label, and mixed with hybridisation buffer.

**Oligonucleotide labeling mixture:**

2μl of T4 kinase buffer
1μl of oligonucleotide 10pmol/μl
2μl of γ-32P ATP (3000Ci/mmol)
Water – up to 20μl
0.5μl (10 Units) of T4 kinase

**Hybridisation buffer (modified version of Church buffer)**

7% SDS
0.5M Sodium phosphate buffer
1 mM EDTA
20xSSC
175.3g NaCl
88.2g Sodium Citrate
Adjust the volume to 1 litre

2.12.5 Extract preparation for ribosomal RNA processing immunoprecipitations.

21 cell cultures were grown to OD₆₀₀ 0.5-0.6, harvested, washed with ice-cold water and snap frozen dry in liquid nitrogen. To disrupt the cells pellets were ground using mortar and pestle in liquid nitrogen until pellet had a consistency of a very fine powder. Following grinding, cell powder was transferred into a tube with Lysis buffer and thawed on ice (1V of cell pellet – 3V of Lysis buffer). Lysate was cleared by centrifugation at 3500 rpm for 5 min, and 14000rpm for 10 min. Extract was collected, and mixed with pre-washed beads. Samples were placed on rotating wheel for 45 min-1 hour at 4°C, after that beads were extensively washed with Lysis buffer, and subjected to RNA extraction. For that 250μl of GTC mix, 150μl of Sodium Acetate (100mM in TE), and 500μl P/C/IAA was added to the beads (or extracts), samples were vortexed, and incubated for 5min at 65°C with shaking. Samples were spun at 14000rpm for 10min at RT. 500μl of the aqueous supernatant was removed and mixed with an equal volume of P/C/IAA, samples were vortexed and spun as described above. 450μl of the supernatant were taken, and precipitated with 1ml of ice-cold Ethanol. RNA was spun down for 30 min at 14000 rpm, obtained RNA pellets were washed with 70% ethanol, dried and resuspended in water. RNA sample was split into two equal parts, one of them was glyoxilated and run on denaturing agarose gel, another half was loaded on 6% polyacrylamide gel as described in detail below. For Total RNA samples RNA from the volume of the extract corresponding to 5% of that used for precipitation was extracted.

Lysis buffer
100mM KCl
20mM Tris pH7.5
5mM MgCl₂
0.2% TritonX100
1mM DTT – freshly added
1 tablet of Complete Roche protease inhibitors
7μl of RNAsein (Promega)
For TAP purifications 100 μl of Rabbit IgG beads (Sigma) were washed trice with 1 ml of lysis buffer. For immuno-precipitations, Protein A Sepharose beads (Amersham) were washed with lysis buffer, and mixed with antibodies and blocking buffer and processed further as described in protocol for spliceosomes.

2.12.6 RNA gel electrophoresis and northern blot analysis.
Low molecular weight RNA species were separated on polyacrylamide gels, and high molecular weight ribosomal RNA species were resolved on 1.2% agarose gel (Sambrook and Russel, 2001). For low molecular weight gels (0.5 mm) Sequagel-6™ (National diagnostics) polyacrylamide was used. Samples were mixed with Formamide loading buffer, incubated for 3 min at 100°C, and chilled on ice. RNA was electrotransferred to Hybond-N or Hybond-N+ membranes for 1 hour at 60 V in 0.5 TBE. After transfer was complete, RNA was UV cross-linked to the membrane once from each side using UV Stratalinker.
For agarose gels corresponding amount of the agarose was dissolved in 1XBPTE (10 mM Pipes, free acid, 30 mM Bis-Tris, free base, 1 mM EDTA, pH 8) buffer, special care was taken to dissolve all the agarose granules.
For sample preparation, 1-2 μl of RNA was mixed with 10 μl of Glyoxal mix (60% DMSO, 20% deionised Glyoxal, 1.2xBPTE, 4.8% Glycerol, 0.2 mg/ml of EtBr) and incubated at 55°C for 1 hour. After that samples were placed on ice for 10 min, and resolved on the gel at 60-65 V for 12-16 hours. After gel run was finished, gel was soaked in 0.2 M NaOH to hydrolyse RNA, followed by two consecutive washes in 6xSSC buffer. RNA samples were transferred to charged HybondN+ membrane by passive capillary transfer; transfer time varied from 4 hours to 12 hours. Following transfer, membrane was UV cross-linked, and subjected to northern blot as described.
2.12.7 Pulse chase analysis.
For pulse chase analysis strains to be labelled were transformed with pRS313 vector, which contains URA3 gene as a nutritional marker. Cell cultures were grown in -Ura medium at all times. For the analysis, appropriate amount of cultures OD$_{600}$ 0.2-0.5 was taken (usually 6ml), and place in 50ml tube on rotating platform. For the pulse, 200μl of tritium labelled Uracil was added to the culture for 1 min. Following the pulse an excess of cold Uracil was added to the culture, at this point sample (1ml) corresponding time-point zero was taken, quick spun, and snap frozen in liquid nitrogen. Samples of the culture were taken at desired time-points and processed as described above.

RNA was extracted and ethanol precipitated as described in 2.12.1. High molecular weight species were resolved on denaturing agarose gel and transferred as described in 2.12.6. Following the transfer and cross-linking, membrane was dried and subjected to autoradiography with LE enhancer screen.

2.13 Protein methods

2.13.1 Purification of Prp43 from E. coli.
PRP43 was cloned into pET16b (Martin et al. 2002). For expression, strain BL21-Codon Plus(DE3)RIL was transformed with the expression vector.

1. Single colony obtained after the transformation was inoculated into 3ml LB +ampicillin +chloramphenicol (stocks are 1,000x) and grown at 37 for a day.
2. At the end of the day the culture was transferred to 500ml of LB + ampicillin +chloramphenicol and grown at 23°C overnight.
3. Following morning expression of the protein was induced by the addition of 1M IPTG to the culture to final concentration 0.1mM.
4. Following induction, culture was grown at 23°C for 3 hours and harvested at 5000 rpm for 10 min.
5. Pellet was resuspended in 10 ml of Buffer A containing protease inhibitors.
6. To lyse the cells Lysozyme (to final concentration 0.2 mg/ml,) and Imidazole (to final concentration 20mM) were added to the suspension. Sample was kept on ice for 30 min.

7. Sample was sonicated to disrupt the cells. Sonicator was set at 38% amplitude and six cycles of 9 sec on, 9 sec off was performed.

8. After sonication Triton X-100 was added to the sample to final concentration 0.1%, subsequently tube was incubated with rotation at 4°C for 30 minutes.

9. Sample was spun in JA25.5 rotor for 15 minutes at 12,000rpm at 4°C.

10. Meanwhile, Ni-NTA agarose beads were prepared. For 10 ml of lysate, 1.5 ml of a 50% slurry of NiNTA agarose (Qiagen) was equilibrated in Buffer A.

11. Cleared lysate was mixed with washed beads and incubated with rotation at 4°C for 45 min-1 hour.

12. Beads were washed trice in Buffer E (5-10 ml per wash). 50 ml tube was used for the first two washes, and then third wash was performed in Bio-Rad econo-column.

13. Protein was eluted using increasing concentrations of Imidazole. Peak fractions were combined and dialyzed versus Buffer D.

Stock solutions for the purification: 1M Tris, pH 7.4, 4M NaCl,1M Imidazole
Buffer A: 50 mM Tris, pH 7.4, 250 mM NaCl, 10% Sucrose
Buffer E: 50 mM Tris, pH 7.4, 250 mM NaCl, 15 mM Imidazole, 10% Glycerol
Buffer D: 50 mM Tris, pH 7.4, 250 mM NaCl, 1 mM EDTA, 2 mM DTT, 0.1% Triton X-100, 10-20% Glycerol

2.13.2 Yeast splicing extract.
The overnight culture was used to inoculate 4-6 litres of an appropriate media. Cells were harvested by centrifugation when the OD_{600} of the culture was 0.7-0.9. Cell pellet was resuspended in 50ml of cold AGK buffer, and spun at 3500rpm for 5 minutes. The pellet was washed one more time with 20ml of AGK buffer. After that, the pellet was resuspended in approximately 0.4 of the volume of AGK buffer, with
DTT added to the suspension to final concentration 2mM. After that the suspension was frozen in liquid nitrogen, and stored at -70°C.

For further processing of the extract frozen suspension was ground with a mortar in liquid nitrogen to a fine powder. Then powder was transferred into polycarbonate centrifuge tubes, thawed on ice and spun at 17000rpm at 4°C for 30 minutes in a Beckman JA 25.50.

The supernatant without lipid layer was transferred to a fresh tube and spun down at 40000 rpm for 1 hour at 4°C. After the spin the supernatant was transferred to dialysis tubing (with MW cut off 10kDA), and placed in 2 litres of cold dialysis buffer for 3 hours at 4°C. Aliquots of the extract was frozen and stored at -70°C.

AGK buffer
For 500ml mix
10ml 0.5M Hepes (pH 7.9)
0.75ml MgCl₂
50ml KCl
50ml Glycerol
Water to 500 ml

2.13.3 Co-immunoprecipitation of proteins from yeast protein extracts.
IgG agarose beads (35μl) were washed three times with NTN. Commercially available conjugated beads were prepared as IgG agarose, but usually 15μl of the beads were taken. Beads were mixed with 30-50μl of 2xIPP buffer and corresponding volume of the splicing extracts and incubated on rotating wheel for 1 hour at 4°C. After the precipitation beads were washed 3 times with NTN, and resuspended in SDS loading buffer. Loaded on a NuPAGE 4-12% gel, and then transferred to PVDF membrane for 2 hours at 32V, blocked in 5% milk in PBST for 1 hour and incubated with appropriate antibodies in PBST/5% milk.

2xSDS loading buffer
125mM TrisCl, pH 6.8
200mM DTT
2.13.4 Preparation of total protein extracts for western immunoblotts.
An overnight culture was (5ml) was inoculated. Next morning OD$_{600}$ of the culture was measured and the volume equivalent of 3 OD$_{600}$ of the cells was spun down. The pellet was resuspended in 0.5ml of freshly prepared 0.2M NaOH and leaved on ice for 10 minutes. 50µl of 50% TCA was added to the sample, mixed, and leaved on ice for additional 10 minutes. Sample was spun down at 14000rpm for 5 min, and the pellet was resuspended in 35µl of Dissociation buffer (100mM Tris pH6.8, 4mM EDTA, 4% SDS, 20% Glycerol, 2% β-Mercaptoethanol, and a little bit of Bromphenol Blue), and 15µl of TrisBase. The samples were heated for 5 minutes at 95°C and loaded on NuPAGE gel, blotted and probed with antibodies as described above.

2.13.5 Glycerol gradient analysis.
Linear glycerol gradients (10-30% v/v) were made using Glycerol Gradient Master (Biocomp). As splicing extract contains 20% Glycerol, splicing extracts were diluted with AGK buffer without Glycerol to reduce final Glycerol concentration. Normally, 120µl of AGK buffer without Glycerol was mixed with 80µl of splicing extract, and layered onto the gradient. Samples were spun for 15 hours at 35000rpm in a SW40 Ti rotor (Beckman). Fractions (400µl) were collected and stored at -80 till further analysis.

2.14 Splicing analysis by Real-Time Quantitative RT-PCR.
This protocol was designed to quantify pre-mRNA, mRNA and lariat RNA species of ACT1 and ASC1 genes, which allows investigate defects in pre-mRNA splicing.
In order to detect pre-mRNA splicing products and intermediates cDNA of RNA species has to be obtained. First, RNA samples were treated with DNase to prevent contamination with genomic DNA, and used for cDNA synthesis. cDNA from pre-mRNA, mRNA and lariat species was specifically made. qPCRs with these cDNAs using specific sets of primers allowed detect variety of RNA intermediates. During qPCR reaction intercalation of SyBr green dye was measured as an increase in fluorescence.

Protocol

1. DNase treatment
For each sample the following components were mixed
12.5μg of RNA
water – to 8μl
1μl of DNaseI buffer
0.2μl of RNAseIn
0.8μl of DNaseI
Samples were carefully mixed and incubated at 37°C for 1 hour, subsequently DNAseI was heat-inactivated at 75°C for 10 min.

2. First strand synthesis
To each sample 2μl of RT primer mix and 2.5μl of water was added, mixed, and 6.25μl of the mixture was transferred to a new tube (for negative no-RT control). Tubes were incubated at 70°C for 5min and then placed on ice to denature RNA. For cDNA synthesis reaction following components were mixed (for each sample)
2μl of 2×cDNA synthesis buffer
0.25μl 0.1M DTT
0.75μl 10mM dNTP mix
0.25μl RNAseIn (Promega)
0.5μl of Thermoscript (Invitrogen) or water for no-RT control
3.75μl of the cDNA mix was added to the denatured RNA, mixed, and incubate for 1-2 hours at 55°C. After that RNAaseA (90μl of 0.1mg/ml stock solution) was added to the reactions and reactions were placed for 1 hour at 37°C.
3. qPCR reactions

Reactions were performed at least in duplicate with no-RT controls.

1. cDNA was diluted a further 1/10 with water (cDNA was diluted in a total of 1/100).

2. For each reaction 5μl of SyBR Green qPCR mix and 1μl of each primer pair (3μM) were mixed (total 6μl). This mixture was pipetted onto 96 microwell plate, then 4μl of cDNA was added to each reaction. The plate was mixed by tapping, spun down and placed into qPCR machine.

![RNA species diagram](image)

**Figure 2.14** Diagram representing RNA species recognized by specific primer sets in qPCR.

PCR program for the *ACT1/ASCI* reporters:

1. 94°C for 2min
2. 94°C 10sec
3. 60°C 10sec
4. 72°C 15sec
5. repeat cycles 2-4 for 50 times
6. 95°C 10sec
7. 60°C 10sec

Plate is read at regular intervals to produce the melt curve.

4. Quantification

Relative abundance of RNA species with respect to a control (RNA from a wild type strain) was calculated using the formula below.

Relative abundance = $2^{\Delta C_t}$, where $\Delta C_t = C_{\text{test}} - C_{\text{control}}$
2.15 Immunofluorescent staining.

For immuno-staining, exponentially growing cells (10ml) were fixed with 1/10 volume of 37% formaldehyde. Tubes were incubated for 40 minutes at RT with constant rotation. Then cells were gently spun down at 3500rpm, RT, for 5 minutes, and washed three times with 1 ml of Buffer B (1.2M sorbitol, 100mM Potassium Phosphate buffer, pH 7). Washed cells were permeabilised in 500μl of spheroplast buffer (Buffer B containing 100mM DTT and 20μl of lyticase (20mg/ml)) on a rotating wheel for 30-45 minutes at 30°C. After that cells were harvested and washed twice with 1ml of Buffer B. Cell pellets were resuspended in 100μl of buffer B and placed on a poly-L-lysine coated slide and left for about 30 minutes for cells to sit. Excess solution was aspirated, and seated cells were blocked with 5% milk in PBST for 20 minutes at RT. Primary antibody was added to the blocking solution, the slide was left overnight in a humid chamber at 4°C. Next morning the slides were washed twice with PBST for 20 min, and covered with PBST with milk and secondary antibody of an appropriate dilution. The slide was placed in a humid chamber for 1 hour at RT, and then washed with PBST as described above. Cells were mounted in DAPI containing vectashield, covered with cover slip, and sealed with nail polish. Slides were examined under the Leica DMR fluorescence microscope; captured images were assembled using image J software.
Chapter 3

Analysis of NTR complex function in splicing

3.1 Introduction

Spp382p is an essential protein and conserved throughout eukaryotic kingdoms. Large-scale purifications identified Spp382p and its human orthologue, TFIP11 protein, with components of the spliceosomal machinery, suggesting that Spp382p is a spliceosomal factor (Boon et al., 2006b; Gavin et al., 2002). Spp382p (also known as Ntr1 for NineTeen Related) was named after its ability to suppress the prp38-1 mutation (Pandit et al., 2006), which has a defect in spliceosome maturation, but the precise mechanism of suppression is not known yet.

Spp382p contains a putative RNA binding motif called G-patch in its N-terminus. G-patch motif is characterised by the presence of six highly conserved glycine residues, which are proposed to mediate RNA binding, however the precise function of the G-patch domain is not determined.

KL Boon confirmed Spp382p association with the spliceosome by showing that Spp382p precipitated preferentially excised intron from in vitro splicing reactions, and associated with U2, U5 and U6 snRNAs in vivo, which indicated that Spp382p is associated with the post-splicing complex. It was established that depletion of Spp382 protein caused accumulation of the excised intron during splicing in vitro and in vivo, suggesting a role for Spp382p in intron metabolism.

Previously several proteins were shown to participate in intron metabolism in S. cerevisiae. First, DEAH box helicase Prp43p was shown to be necessary for the release of the excised intron from the post-splicing complex after completion of the splicing reaction (Martin et al., 2002). ATPase deficient mutants of Prp43 caused accumulation of the excised intron lariat with the post-splicing complex, therefore preventing its degradation. After intron release, the 2'-5' phosphodiester bond of the lariat undergoes cleavage by de-branching enzyme Dbr1p (Ruskin and Green, 1985),
which makes it a suitable substrate for degradation 5'-3' by Rat1 and Xrn1, or 3'-5' by exonucleaseolytic activity of the exosome (Coller and Parker, 2004; Mitchell and Tollervey, 2001). Mutation of the non-essential DBR1 caused accumulation of the excised introns (Chapman and Boeke, 1991). However in the case of Spp382p it was not clear whether inton accumulation was due to the failure of intron degradation process, or a defect of spliceosome disassembly.

Here I present evidence in support of the latter hypothesis, showing that in Spp382-depleted extracts spliceosome disassembly is impaired, and excised inton is still associated with the post-splicing complex. Additionally I demonstrate requirement of Spp382p for stable association of the excised inton release factor Prp43p with the post-splicing complex.

In large-scale characterisation of yeast ORFs with unknown functions YkO22cp (later renamed Ntr2p) was co-purified in the complexes containing Spp382p (Gavin et al., 2002). Additionally, Ntr2p was shown to interact with Spp382p in yeast two-hybrid (Gavin et al., 2002). All this taken together strongly suggested that Ntr2p might be a newly discovered splicing factor. In this chapter I investigate Ntr2p association with the spliceosome and show Ntr2p association with the post-splicing complex, confirming that Ntr2p is indeed a new splicing factor.
3.2 Depletion of Spp382p affects spliceosome disassembly

In order to investigate the function of Spp382p, KL Boon constructed a strain with gene SPP382 under control of the regulatable GAL1 promoter. On switching to repressing (glucose containing) medium, rapid depletion of the protein from the cells occurs, allowing study of the defect caused by the absence of the protein, and therefore identification of the step of the process where the protein of interest might be involved. It was established that upon depletion of Spp382p from the cells, there is an accumulation of the excised intron in vivo and in vitro, suggesting that intron metabolism was affected (Boon et al., 2006a).

I immunoprecipitated Prp8p from an in vitro splicing reaction and analyzed the precipitated products. The in vitro splicing assay is based on cell-free extract, pre-mRNA substrate radiolabelled by in vitro transcription; using this assay allow monitoring of splicing activity and analyse individual components of spliceosomal machinery. An important advantage of this assay is its ability to easily distinguish between the products and intermediates of the splicing reaction by the speed of their migration in the gel. In the following experiments ACT1 pre-mRNA was used as a substrate for in vitro splicing assay.

Prp8p is a U5 snRNP component, and core spliceosomal protein and co-precipitates all the intermediates and products of in vitro splicing, including the excised intron under normal conditions. Therefore, repeating the assay under Spp382-depleted conditions would allow to distinguish between a general disassembly defect, or a defect in de-branching of the excised intron.

Strain BK4H with SPP382 under control of P\text{\_GAL1} promoter was pre-grown in YPGR medim, and than transferred to YPDA medim for 10 hours for the depletion of Spp382p. Strain BMA38a (isogenic wild type) was treated as BK4H and used as wild-type control. Splicing extracts from the frozen cell pellets were prepared as described in 2.13.2, and 50µl in vitro splicing reactions were assembled with normal and Spp382-depleted extracts. Reactions were immunoprecipitated with anti-Prp8
antibodies as described in 2.12.4. After the immunoprecipitation samples were washed extensively, subjected to RNA extraction, loaded on PAGE gel and analysed by autoradiography.

Figure 3.1. Co-immunoprecipitation of Prp8p with the products of *in vitro* splicing reaction. Splicing reactions (50µl) were assembled using normal and Spp382-depleted extracts with *ACT1* pre-mRNA as substrate. Reactions were mixed equal volume of 2XPPT buffer and beads with pre-bound anti-Prp8 antibodies or beads incubated with pre-immune serum (P1). Immunoprecipitations were carried out for 1 hour at 4°C, subsequently beads were washed extensively with NTN buffer, and subjected to RNA extraction. For Unbound fraction RNA was extracted from 10% of the supernates after immunoprecipitation. RNA species were analysed by PAGE (7%) and autoradiography.
As can be seen in figure 3.1, in splicing reaction assembled with Spp382p-depleted extract there is an accumulation of the excised intron and decrease in production of mRNA (lane 3). This implies that Spp382p affects spliceosome disassembly and recycling. Under splicing conditions Prp8p is associated with the excised intron-lariat, which indicates that excised intron is associated with the post-splicing complex and stays inaccessible for the debranching activity of Dbr1p.

**3.3 Ntr2 protein is associated with the post-splicing complex.**

In order to investigate the putative function of Ntr2p in pre-mRNA splicing, physical association of Ntr2p with the spliceosome was tested. First, the ability of TAP-tagged Ntr2p to co-precipitate spliceosomal snRNAs was investigated. Splicing extracts from strains containing TAP-tagged Ntr2 (BK2T), TAP-tagged Spp382(BK4T) and an untagged strain (BMA38a) were prepared. Spp382-TAP was used as a positive control, as it has been established that it co-precipitates U2, U5 and U6 snRNAs (Boon et al., 2006a). snRNAs precipitation was performed as described in 2.12.4, and results are presented in figure 3.2.

As can be seen in figure 3.2, Ntr2p, like Spp382p, co-precipitates U2, U5, and U6 snRNAs, which indicates that Ntr2p is indeed associated with the spliceosome. The level of the precipitation of U1 snRNA is the same as in non-tagged strain, suggesting that Ntr2p does not precipitate U1 snRNA. U4 snRNA is also absent from the precipitate indicating that Ntr2p is not associated with complex B. This suggests that Ntr2p is associated with the catalytic spliceosome and/or post-splicing complex as these complexes consist of U2, U5 and U6 snRNAs.

In order to determine on which stage of splicing Ntr2p is associated with the spliceosome precipitation of the products of an *in vitro* splicing reaction was performed. TAP-Prp8 (BK8T) and TAP-Spp382 (BK4T) were employed as
positive controls, as it is known that Prp8p is associated with all the intermediates and products of in vitro splicing reaction and KL Boon reported that Spp382p precipitates presumably excised intron from in vitro splicing reactions. Non-tagged BMA38a strain was used as a negative control for background level. Additionally, as Ntr2p was reported to interact with Spp382p in a yeast two-hybrid screen and not with any other splicing factor (Gavin et al., 2002), it was conceivable that Spp382p is required for the association of Ntr2p with the spliceosome. To test this, Ntr2-TAP strain with SPP382 under control of glucose-repressible GAL1 promoter was used (BK4H2T). Pre-culture of BK4H2T was grown in YPGR media, then transferred to YPDA for depletion for 10 hours. Growth of the BK4H2T is severely impaired with doubling time of approximately 4 hours (KL Boon’s thesis). To grow culture for the depletion, appropriate volume of pre-culture was spun down, resuspended in YPDA medium and inoculated in corresponding volume of pre-warmed YPDA medium. Splicing reactions (50μl) were assembled and precipitated with rabbit IgG agarose beads, washed extensively, and loaded on the gel. Dried gel was subjected to autoradiography. As shown in figure 3.2, Ntr2p efficiently precipitates excised intron from in vitro splicing reactions, and pre-mRNA and mRNA on a level slightly above background (Non-TAP precipitation, lane 6). This result suggests that Ntr2p is presumably associated with intron-containing post-splicing complex, and might be weakly associated with catalytic spliceosome. Interestingly, Ntr2p also precipitates excised intron and pre-mRNA from Spp382-depleted extract, indicating that Spp382p is not necessary for Ntr2p association with the post-splicing complex. This finding suggests that Ntr2p has other unidentified interactions within the spliceosome.

In order to investigate the function of Ntr2p, NTR2 was placed under control of P_{GAL1} promoter. The strain was verified by yeast-colony PCR and western blot. Depletion of Ntr2p was confirmed by western blot with the sample obtained after 10 hours after shift to YPDA medium. Despite that NTR2 is an essential gene, depletion of the Ntr2p using this system does not affect cell viability and growth, probably due to some residual expression of NTR2. However, the lack of the
phenotype in Ntr2-depleted culture prevented studying of the effect of Ntr2 depletion on splicing.

Figure 3.2. Ntr2p is associated with snRNAs. Splicing extracts (50 μl) prepared from non-tagged strain (BMA38), or TAP-tagged Spp382 or Ntr2 were mixed with equal volume of 2xIPP buffer, and 30μl of rabbit IgG agarose extensively washed with 1xIPP buffer. Samples were incubated for 2 hours at 4°C on rotating wheel. Beads were washed in NTN buffer, RNA was extracted from the beads and analysed by northern blotting using specific probes for U1, U2, U4, U5 and U6 snRNAs. Total RNA (Input) was extracted from 12.5μl of splicing extract.
Figure 3.3. Ntr2p is associated with the excised intron, and does not require Spp382p for its association with the post-splicing complex.

Splicing reactions (50 µl) were assembled using Prp8-TAP, Spp382-TAP, Ntr2-TAP, Ntr2-TAP Spp382-depleted and non-tagged strain BMA38a as a negative control. Reactions were incubated for 30 min at RT, mixed with equal volume of IPP2x buffer and 30µl of pre-washed rabbit IgG agarose beads. Samples were incubated on rotating wheel at 4°C for 1 hour, RNA was extracted, resolved on 7% PAGE gel and subjected to autoradiography.
3.4 Spp382p and Ntr2p co-sediment in Glycerol Gradients.

To investigate Ntr2p distribution by glycerol gradient centrifugation strain with TAP-tagged Ntr2 P<sub>GAL1:3HA</sub>-Spp382 (BK4H2T) was used. In order to test co-distribution of Ntr2p and Spp382p, splicing extract was prepared from the strain BK4H2T grown in YPGR medium. To test the effect of Spp382-depletion on Ntr2p distribution, strain BK4H2T was pre-grown in YPGR medium, and then transferred to YPDA medium for depletion as described above. For glycerol gradients splicing extracts (80μl) were diluted with AGK buffer without glycerol (120μl), and loaded on 10-30% (v/v) glycerol gradients. After centrifugation 400μl fractions were collected, and alternate fractions were immunoprecipitated with IgG agarose as described in 2.13.3, samples were resolved on 4-12% gel, blotted and probed with PAP (for TAP-tag detection) and anti-HA antibodies.

Ntr2p and Spp382p co-sediment in glycerol gradient with the peaks at the bottom of the gradient (fractions 1-3) and in the fractions 17-23 (figure 3.4A). The peak at the bottom of the gradient likely represents large splicing complexes, while the peak in the low density fractions 17-23 probably represents Ntr2p/Spp382p complex, which is not associated with the spliceosome. Noticeably, only in the top fractions of the gradient Spp382p is represented by two bands, a phenomenon initially observed by KL Boon. He suggested that as the human orthologue of Spp382 TFIP11p is heavily phosphorylated (Wen et al., 2005), the lower band might be unmodified form of the protein, however the possibility that it is a degradation product cannot be excluded. On next stage, distribution of Ntr2p in Spp382-depleted extract was tested. Presumably, if the depletion of Spp382p protein disrupts Ntr2p association with the spliceosome, a shift in Ntr2p distribution could be observed. As shown in figure 3.4B depletion of Spp382p does not affect distribution of Ntr2p, supporting the in vitro splicing results that Spp382p is not necessary for Ntr2p association with the spliceosome.
Figure 3.4. Distribution of Ntr2p and Spp382p.

Extracts prepared from BK43.6 Spp382p is necessary for Prp43p association with the spliceosome

DEAH box helicase Prp43p was shown to be necessary for the release of the excised intron from the post-splicing complex (Martin et al., 2002). Additionally, Prp43p was shown to interact with Spp382p in a yeast two-hybrid screen (Lebaron et al, 2005). Spp382p was the only splicing factor reported to interact with Prp43p, therefore possibility that Spp382p is necessary for the Prp43p association with the spliceosome was tested.

As Prp43p was believed to associate with the spliceosome only transiently, it was decided to employ an ATPase-deficient dominant negative mutant Prp43T123A in order to stall complexes with bound Prp43p. Mutation in Walker motif A drastically impairs the ATPase activity of Prp43p, and blocks the release of the excised intron from the post-splicing complex (Martin et al., 2002). Plasmid expressing His7Prp43T123A in pET16b (pET16-prp43T123A) was the gift of Dr B. Schwer. BL21-Codon Plus (DE3)RIL strain of E.coli was transformed with the plasmid, and recombinant protein was affinity purified as described in 2.13.1. Elution and purity
of the sample was confirmed by Coomassie staining. Subsequently recombinant protein was added to an *in vitro* splicing assay as described in (Martin et al., 2002).

To determine whether the depletion of Spp382p affects Prp43p association with the spliceosome, immunoprecipitation of Prp43p from an *in vitro* splicing reaction was performed from normal and Spp382-depleted extracts. To stall the spliceosomes in post-splicing complex, 1μg of recombinant Prp43T123A protein was added to each *in vitro* splicing reaction (50μl). Samples were processed as described in 2.12.4. As shown in figure 3.5, lane 5 depletion of Spp382p impairs Prp43p association with the post-splicing complex.

In order to confirm that Prp43p fails to co-precipitate excised intron due to the absence of Spp382p, and not due to a secondary effect, a reconstitution experiment was performed. As previous attempts to reconstitute Spp382-depleted extract using recombinant Spp382 protein proved to be unsuccessful probably because of the nature of the protein (recombinant preparations of proteins with nucleic acid binding domains often contain RNA and RNAases from *E.coli* which could interfere with the assay), Spp382p was affinity purified from yeast splicing extract. Strain BK4H over-expressing *SPP382* from *P_{GAL1}* promoter was used for affinity purification of Spp382p. To prepare reconstituted extract, affinity purified protein was eluted from the beads, and mixed with Spp382-depleted extract. Reconstituted splicing extract was used for *in vitro* splicing assay as described above. Results of the reconstitution are presented in figure 3.5, lane 6. It is clear that addition of affinity purified Spp382p restored the ability of Prp43p to precipitate excised intron from *in vitro* splicing reaction, which indicates that Spp382p aids in recruitment of Prp43p to post-splicing complex.
Figure 3.5 Spp382p is necessary for Prp43p association with the post-splicing complex.

50 μl splicing reactions were assembled with normal, Spp382–depleted and reconstituted extracts. Reactions were supplemented with recombinant dnPrp43T123A, and processed as usual. Anti-Prp43 antibodies were used for the immunoprecipitation. For affinity purified Spp382p, strain BK4H over-expressing SPP382 from P_GAL1 promoter was used. Extract prepared from the strain grown in YPGR media (20μl) was used for immuno-precipitation with 15μl of anti-HA agarose beads, on the next step beads were extensively washed with NTN buffer. Two last washes were performed in 1xsplicing buffer. Subsequently beads were mixed with 10μl of 1xsplicing buffer, and 0.5μg of HA-peptide (Sigma) was added to the sample for the competitive elution of Spp382p from the beads. Elution was performed for 8 hours at 4°C, after that 5μl of the superNAtant was mixed with 100μl of Spp382-depleted splicing extract, and incubated for 30 min at 4°C to promote incorporation of purified Spp382p into the spliceosomal particles. Reconstituted splicing extract was used for in vitro splicing assay together with Spp382-depleted extract and non-depleted extracts.
3.7 *Spp382p, Ntr2p and Prp43p are associated with catalytic spliceosomes.*

Spp382p, Ntr2p and Prp43p are predominantly associated with the excised intron *in vitro* splicing reaction, but these proteins also precipitated little amounts of pre-mRNA and mRNA from *in vitro* splicing, which suggested that Spp382p, Ntr2p and Prp43p might be associated with the catalytic spliceosomes. To further investigate this issue, aberrant *ACTI* pre-mRNA truncated just upstream of the 3’SS was used. Spliceosomes assembled on this substrate undergo first step, but are unable to perform second step of splicing, therefore using this substrate for *in vitro* splicing causes accumulation of catalytic spliceosomes.

Splicing reactions (50μl) with non-tagged extract (BMA38a), Prp8-TAP (BK8T), Spp382-TAP (BK4T), Ntr2TAP (BK2T), Spp382-depleted Ntr2TAP (BK4H2T), and Prp43TAP (BK43T) with aberrant substrate were assembled, and processed as described in 2.12.4. Result is presented in figure 3.6.
Figure 3.6. Spp382p, Prp43p, and Ntr2p are associated with catalytic spliceosomes. Aberrant substrate (Δ3'SS) was used for *in vitro* splicing reaction. Splicing reactions (50 μl) were assembled, incubated for 30 min at RT, and precipitated with rabbit IgG agarose. RNA extracted from beads and 20% of unbound fractions was loaded on 7% PAGE gel, gel was dried and subjected to autoradiography.

Results of this experiment indicate that Spp382p, Ntr2p and Prp43p are likely to be associated with catalytic spliceosomes as Spp382p, Ntr2p and Prp43p efficiently precipitate aberrant lariat and exon1 from *in vitro* splicing reaction with aberrant substrate. Interestingly, while Prp8p precipitates approximately equimolar amounts of aberrant lariat and exon1, Spp382p, Prp43p and Ntr2p show preferential precipitation of the aberrant lariat. This suggests that under conditions of the precipitation these proteins are more stably associated with the lariat species.
Strikingly, Ntr2p shows only background precipitation of the aberrant intermediate of the splicing reaction in Spp382-depleted extract, suggesting that Spp382p is required for Ntr2p association with the catalytic spliceosome. As Ntr2p is still associated with normal excised intron-lariat in the absence of Spp382p, this finding might point to extensive protein-protein re-arrangements within the spliceosome between catalytic steps.
3. 8 Discussion

In this chapter I present evidence that depletion of Spp382p causes a spliceosome disassembly defect, as it appears that Prp8 is accumulated in a complex with the excised intron. In vivo Prp8p is found to be associated with an increased amount of U2, indicating accumulation of Prp8 in aberrant post-splicing complexes (Boon et al., 2006a).

Large-scale yeast two-hybrid screens previously identified interaction between Ntr2p and Spp382p (Ito et al., 2001; Uetz et al., 2000). I present data that Ntr2p is a newly identified splicing factor as it is physically associated with the post-splicing complex. Ntr2p precipitates excised intron from in vitro splicing reaction, and U2, U5 and U6 snRNAs, which suggests a role for the protein in spliceosome disassembly. Additionally, it appears that Spp382p is not required for Ntr2p association with the post-splicing complex as in Spp382-depleted extract Ntr2p is still associated with the excised intron. This finding indicates that Ntr2p has unidentified interactions within the spliceosome. Indeed, when the work on this thesis was in progress, a paper on the subject was published. It showed that Ntr2p interacts with DExD/H box helicase Brr2p in yeast two-hybrid assay, and suggested that Ntr2p acts as a receptor to bring Spp382p to the spliceosome (Tsai et al., 2007).

Analysis of sedimentation profiles of Ntr2p and Spp382p established that Ntr2p and Spp382p co-distribute in glycerol gradients with distinctive peaks in the top and in the bottom of the gradient. This result indicated that Ntr2p and Spp382p likely co-exist within high and low molecular weight complexes. While bottom fractions are likely to represent various high molecular weight spliceosomal complexes, it is not entirely clear what the top peak represents. It was suggested that the lower-density peak might be a Ntr2-Spp382p dimer, which is not snRNP-associated (Boon et al., 2006a). Previously it was established that Spp382p co-sediments with Prp43p, which suggests that Prp43p, Spp382p and Ntr2p might form a complex (Boon et al., 2006a). Moreover, based on yeast two-hybrid and biochemical studies it was proposed that Ntr2p, Spp328p and Prp43p form a trimer (Tsai at al., 2005).
Interestingly, the only other protein found in this snRNP-free part of the gradient is the DExD/H box helicase Brr2p (mature U5 snRNP component). Brr2p is proposed to leave U5 snRNP or replaced by Aar2p during spliceosome recycling, and it is not clear whether Brr2p stays associated with other proteins after it leaves U5 snRNP (Boon et al., 2007). As Ntr2p interacts with Brr2p in a yeast two-hybrid assay (Tsai et al., 2007), it is possible that Brr2p, Ntr2p, Spp382p and Prp43p form a subcomplex following spliceosome disassembly. Further experiments are required to support this hypothesis.

Association of Prp43p with the spliceosome is very weak, and barely detectable in vitro splicing reactions assembled with normal substrates and normal splicing extracts (Martin et al., 2002; Tsai et al., 2005). Prp43T123A mutant blocks the release of the excised intron-lariat from the post-splicing complex (Martin et al., 2002), and is used to study Prp43p association with the post-splicing complex. In this chapter I show that Spp382p is required for the association of the Prp43pT123Ap with the post-splicing complex. In Spp382-depleted extract Prp43T123Ap does not precipitate excised intron from in vitro splicing reaction, however addition of the affinity purified Spp382p restores the association of the Prp43T123A with the excised intron. Interestingly, Prp43p also co-precipitates pre-mRNA from in vitro splicing reaction on a level above background, which might indicate that Prp43p is present in early spliceosomal complexes. Significantly, human Prp43p is a U2 snRNP-associated protein (Dybkov et al., 2006), which might suggest that Prp43p is associated with the spliceosome at all stages of splicing as a part of U2 snRNP, but its activity is required for the spliceosome disassembly. Additionally human orthologue of Spp382p, TFIP11 protein (for tuftelin-like protein), is found in tri-snRNPs, suggesting that Spp382p might join the spliceosome as a part of tri-snRNP. Indeed, Spp382p, Ntr2p and Prp43p are associated with in vitro assembled catalytic spliceosomes stalled after first catalytic step, supporting the idea that these proteins might associate with the spliceosomes before disassembly step.
Chapter 4

Spp382 is a pre-rRNA processing factor

4.1 Introduction

In addition to its association with splicing factors, the helicase Prp43p was co-isolated with multiple pre-ribosomal particles (Dez et al., 2002, Lebaron et al., 2005, Combs et al., 2006, Leeds et al., 2006), including precursors of both small and large ribosomal subunits. However, the precise function of Prp43p in pre-rRNA processing and the stage where it acts remains to be determined.

Spp382p and Ntr2p were shown to be accessory factors for DEAH-box helicase Prp43p in splicing (Tsai et al., 2005; Boon et al., 2006; this thesis), and co-factors for Prp43p in pre-rRNA processing have not been identified. This led to suggestion that Spp382p and Ntr2p might assist Prp43p in pre-rRNA processing as well as in splicing. In this chapter I present evidence that Spp382p, but not Ntr2p, is indeed associated with pre-rRNA particles and plays a role in rRNA biogenesis.

4.2 Depletion of Spp382p causes a defect in pre-rRNA processing

It was previously established that depletion of Spp382p affects cell growth (Boon et al., 2006). However, it was also established that depletion of Spp382p causes only a mild splicing defect, and it was proposed that Spp382p might have an essential function different from splicing (Boon et al., 2006). Since Prp43p was implicated in both splicing and pre-rRNA processing, the effect of Spp382-depletion on pre-rRNA processing was investigated.

In order to study the effect of Spp382-depletion on pre-rRNA processing, strain BK4H was pre-grown in YPGR, transferred to YPDA medium, and aliquots of the culture maintained in early log phase were taken after 0, 2, 4 and 6 hours. Depletion of Spp382p was confirmed by western blot (Fig 4.1 C). For positive control culture of BMA38a strain was treated in the same way. RNA was extracted from the
samples using the GTC/Phenol method, and steady state levels of pre-RNA precursors and mature species were analyzed by northern blot (figure 4.1 A and B).

Figure 4.1. Depletion of Spp382p has a rapid effect on pre-rRNA processing. BMA38a and BK4H strains were grown in YPGR, and transferred to YPDA. At the time-points given, samples of the cultures were taken, and total RNA was extracted. RNA was resolved on 1.2% agarose gel (large molecular weight species, panel A) and 6% (w/v) polyacrylamide gel (small molecular weight species, panel B). RNA was transferred to NylonN+ membrane, and was subjected to northern blot analysis using various probes. Depletion of Spp382 was confirmed by western blot analysis (Panel C). Samples for western were prepared as described in 2.13.4 and loaded on 4-12% gel, blotted and probed with HRP conjugated anti-HA antibodies (1:3000).
Result presented in figure 4.1 indicates that depletion of Spp382p affects pre-rRNA processing. Thus, after two hours in YPDA there is an accumulation of 35S and 23S precursors, and a decrease in a level of 20S, which suggests that cleavages at Ao, A₁ and A₂ are affected (figure 4.1A). Decrease in the level of 20S indicates a defect in mature 18S processing. At the same time, levels of 27SB and 7S appear unchanged, suggesting that depletion of Spp382p does not affect large subunit processing, and has a primary effect on the small subunit biosynthesis. Levels of mature 25S, 18S, and 5.8S also remain unchanged, however taking into consideration the short time frame of the experiment (6 hours), it is likely that the defect on the level of the mature species is not yet apparent, due to the slow turnover of mature rRNAs. Additionally, no change in the level of mature U3 snoRNA is observed, indicating that at Ao, A₁, A₂ defect can not be explained by U3-depletion. In support that Spp382-depletion only causes mild splicing defect, there is a very modest accumulation of pre-U3 snoRNA after 4 hours of depletion (figure 4.1 B). These data suggest that the defect in splicing caused by Spp382-depletion, cannot solely account for the defect in pre-rRNA processing, and indicate that Spp382p might play a direct role in pre-rRNA processing.

Pulse-chase analysis was performed to investigate the kinetics of pre-rRNA processing in the Spp382-depleted strain. BMA38a and BK4H strains carrying empty pRS313 vector (as uracil uptake and its incorporation into pre-rRNA was studied) were pre-grown in −ura +gal medium, and transferred to −ura+glucose medium for 4 hours to deplete Spp382p. Pulse labelling was performed with ³H-uracil, and excess of non-labelled uracil was added for the “chase” (described in 2.12.7). Samples were collected, and processed as described in 2.12. 7. Results are presented in figure 4.2.
Figure 4.2 Pulse-chase analysis of Spp382-depleted strain shows defects in pre-rRNA processing. Pulse labelling was performed with $^3$H- Uracil for 1 min, then excess of unlabelled uracil was added to the culture. Samples were collected at indicated times after unlabelled uracil addition. RNA was extracted from the samples, separated on 1.2% (w/v) agarose gel, blotted and subjected to autoradiography.

Analysis of the labelled RNA from the pulse-chase experiment shows a significant delay in maturation of 35S, which accumulates present at later time points in the Spp382-depleted strain. Additionally, processing of both 27SA/B and 20S is delayed as no mature species are produced in the mutant strain. The results suggest that there is a severe pre-rRNA processing defect in Spp382-depleted strain, which affects biosynthesis of small and large subunits.

As depletion of Ntr2p did not show any effect on cell viability, effect of Ntr2p depletion on pre-rRNA processing was not assessed.
4.3 Spp382p is associated with pre-rRNA precursors.

To establish whether Spp382p and Ntr2p take part in pre-rRNA processing, physical association of Spp382p and Ntr2p with pre-rRNA particles was investigated. For this, affinity purification of these proteins was performed based on the Protein A moiety of the TAP tag (first step only), and precipitates were tested for the presence of pre-rRNA precursors.

TAP-tagged Spp382p (KL4T), Ntr2p (KL2T) strains were used, with non-tagged strain (BMA38a) as a negative control, and TAP-tagged Prp43p (KL43T) as a positive control. Extracts for purification were prepared as described in 2.12.5 from 21 cultures and mixed with IgG agarose beads. After co-precipitation, RNA was extracted, loaded on the gel and analysed by northern blot.

As shown in figure 4.3, Spp382p, but not Ntr2p precipitates pre-rRNA precursors. The level of the precipitation of pre-rRNA precursors by Spp382p is similar to that of Prp43p, which was previously reported to be weakly associated with pre-rRNA precursors. Levels of 35S, 32S, 20S, 27SA/B and 7S were enriched in the Spp382p precipitate, suggesting that Spp382p, like Prp43p, is associated with the precursors of both small and large ribosomal subunits. Intriguingly, Spp382p, like Prp43p, precipitates 20S and mature cytoplasmic 18S on a level above background. As conversion of 20S to 18S occurs in the cytoplasm, this result suggests that Spp382p might be associated with the late-40S particles. The significance of this association remains unknown.
Figure 4.3. Spp382p, but not Ntr2p precipitates pre-rRNA precursors. IgG agarose beads (50μl) were washed with lysis buffer and mixed with extracts prepared from 2l of exponentially growing culture. Co-immunoprecipitation was performed for 1 hour at 4°C on rotating wheel. Beads were washed extensively and subjected to RNA extraction. “Total RNA” extracted from the cell lysate corresponded to 1% of that used for immuno-precipitation served as Total sample. Half of the samples were glyoxal-denatured and resolved on 1.2% agarose gel, while another half was loaded on 6% PAGE gel. After the transfer membranes were hybridized with variety of probes to multiple RNA species.
4.4 Discussion

In this chapter I show that Spp382p, like Prp43p is associated with the precursors of rRNA processing, and depletion of Spp382p affects pre-rRNA processing, which suggests that Spp382p might be a new pre-rRNA processing factor.

Depletion of Spp382p causes substantial accumulation of 35S and 23S after 2 hours in YPDA medium, and a decrease in 20S, which suggests that A0, A1 or A2 cleavages might be affected, which is also consistent with the results of the pulse-chase experiment.

As Spp382p is also implicated in splicing, there is a likely argument that the observed defect in pre-rRNA processing is secondary. U3 snoRNA is implicated in A0, A1 and A2 cleavages, and depletion of U3 due to the splicing defect will have a phenotype similar to that of Spp382-depletion. Additionally, a large proportion of genes coding ribosomal proteins are intron-containing, which suggests that defective splicing of ribosomal protein transcripts might cause a secondary effect on pre-rRNA processing. However, there are several observations that favour a direct effect of Spp382 on pre-rRNA processing. First, depletion of Spp382p has no effect on the level of mature U3 snoRNA and causes only mild accumulation of pre-U3 after 4 hours in YPDA. This eliminates the possibility that drastic effect of Spp382-depletion on pre-rRNA processing is due to the lack of U3 snoRNA.

It was suggested that assembly of ribosomal proteins on pre-rRNA probably provides a platform for the pre-rRNA processing enzymes, and therefore correct assembly of ribosomal proteins on pre-rRNA is necessary for pre-rRNA processing events to take place (Ferreira-Cerca et al., 2005), therefore it is conceivable that Spp382-depletion affects pre-rRNA processing through deficiency of r-proteins. However, it was shown that depletion of Spp382p does not cause significant reduction of any mRNA coding ribosomal proteins (Boon et al., 2006), suggesting that it is highly unlikely that the defect in pre-rRNA processing is due to the lack of ribosomal proteins. Additionally, analysis of the splicing arrays suggests that ribosomal genes form a regulon, which might indicate a common regulatory mechanism for genes coding r-proteins (Pleiss and Guthrie., 2007). It was reported that mRNAs, coding r-proteins
have long half-lives, and splicing defects affect the level of r-mRNAs proportionally to the level of the splicing defects (Pleiss and Guthrie, 2007), supporting the that a defect in r-proteins production cannot solely cause the observed defect in pre-rRNA processing. All the above supports the idea that Spp382p is directly involved in pre-rRNA processing.

The TAP affinity purification experiment reveals that Spp382p, but not Ntr2p, co-precipitates pre-rRNA precursors, which indicates physical association of Spp382p with the pre-rRNA processing machinery (or with pre-ribosomes). Moreover, as Spp382p repeats the pattern of precipitation of pre-rRNA precursors by Prp43p, it suggests that Prp43p and Spp382p may act together in pre-rRNA processing as well as in splicing.

Spp382p, like Prp43p, is associated with the pre-rRNA precursors rather weakly. It is believed that DExD/H-box helicases have very weak and/or very dynamic association with the spliceosome and pre-rRNA processing machinery. In a view of the chaperone-like function of DExD/H-box helicases, it is conceivable that their activity is required for a certain step, after which the enzyme dissociates from the substrate(s) and gets recycled. Therefore, at a given moment in time there is only a small sub-population of the spliceosomes or pre-ribosomes that is associated with a particular helicase, and also this interaction by definition is very transient. A recent paper, in which splicing competent catalytic complexes were isolated, divided splicing proteins into "core" and "top" sub-groups (Bessonov et al., 2008). DExD/H-box helicases, including Prp43p and its accessory factor Spp382p, belong to the "top" group, which is easily dissociated from the "core" spliceosome. Applying similar logic to pre-rRNA processing machinery, Prp43p and the rest of DExD/H-box helicases of pre-rRNA processing, the majority of RNA modifying enzymes and nucleases would constitute the putative "top" group of the pre-rRNA processing machinery, which \textit{ab initio} has weak/transient association with the "core" machinery. Thus, several known pre-rRNA processing factors proved to be elusive to isolate with pre-RNA processing machinery. G-patch protein Gno1p interacts with Prp43p and is proposed to be an accessory factor for Prp43p in pre-rRNA processing (Lebaron et al., 2005). Deletion of Gno1p results in pre-rRNA processing and
snoRNA maturation defects (Guglielmi and Werner, 2002). However, Gno1p failed to be co-purified with pre-rRNA precursors. Therefore, despite weak association of Spp382p and Prp43p with splicing and pre-RNA processing machineries, these proteins might play an important function in both processes. The remarkable fact that Spp382p and Prp43p are shared subunits between two machineries probably has some functional relevance. It is very tempting to propose that Spp382p and Prp43p play a role in a concerting mechanism between the two processes, however further experiments are needed to prove this.
Chapter 5

Role of Prp43 protein in splicing

5.1 Introduction

Multiple reports have placed yeast protein Prp43p within the post-splicing complex, which was confirmed by Prp43p association with U2, U5, U6 snRNAs, and excised intron (Martin et al., 2003; Lebaron et al., 2005; Leeds at al., 2006; Combs et al., 2006; Small et al., 2006). Despite that Prp43p is required for spliceosome disassembly, the target of Prp43p activity in the process remains unknown. In this chapter I show that Prp43p might play a role in U6 snRNA release from the post-splicing complex.

In chapter 3 I showed that Prp43p is associated with catalytic spliceosomes stalled after the second step of splicing. Interestingly, Prp43p precipitates significant amount pre-mRNA from Spp382-depleted extract from an in vitro splicing reaction, suggesting that Prp43p might be associated with the pre-catalytic complex. In support of this hypothesis, the human orthologue hPrp43 was found to be U2 snRNP-associated (17S), and was also co-purified with complex A (Behzadnia et al., 2006; Deckert et al., 2006; Dybkov et al., 2006). Additionally, hPrp43p was shown to co-precipitate U2 and U1 snRNAs, indicating that hPrp43p may be associated with early spliceosomal complexes (Fouraux et al., 2002). In this chapter I present evidence that the yPrp43 protein is likely to be associated with the spliceosome as early as Complex A.

All DEAH-box proteins have a conserved helicase core domain and unique N- and C-terminal regions. It was shown that the C-terminus of Prp43p is not required for the ATPase activity of Prp43p in vitro, but is essential for cell viability (Tanaka et al., 2007). Additionally, the C-terminus of Prp43p interacts with Spp382p (Tsai et al., 2005). The role of this interaction is unknown, as Spp382p also interacts with the helicase core of Prp43p, and this interaction is enough to activate ATPase activity of
Prp43p in vitro (Tanaka et al., 2007). Here, I demonstrate that a mutation in the C-terminus of Prp43p causes a defect in splicing, but not in pre-rRNA processing. I propose that the C-terminus is likely to play a role in regulation of the ATPase activity of Prp43p within the post-splicing complex.

5.2 Prp43 protein is associated with Complex A.

In order to investigate the possibility that Prp43p is a part of pre-catalytic spliceosomal complexes, the association of Prp43p with splicing complexes was assayed in Prp8-depleted yeast splicing extract that is unable to form Complex B. It was previously reported that depletion of Prp8p destabilizes U5 snRNP, and therefore affects tri-snRNP formation and formation of Complex B (Brown and Beggs, 1992). Therefore, this splicing extract will form Complexes E and A, and potentially aberrant B complex, but not catalytically competent spliceosomes. Strain KL3 (P_{GAL1}PRP8) was used for the preparation of Prp8-depleted extract. First, growth defect of the culture of KL3 strain was assessed following shift to YPDA media. Depletion of Prp8p severely affects growth of the culture (figure 5.1), suggesting that Prp8p is efficiently depleted, and strain KL3 can be used for the further experiments.
Figure 5.1. Depletion of Prp8p causes a severe growth defect. Strain KL3 and the isogenic wild type, BM38A, were pre-grown in YPGR, and than transferred to YPDA. Optical density of the culture was measured every two hours.

Splicing extracts were prepared from strains KL3 and BM38A 6 hours following transfer into YPDA, and were checked for their splicing activity *in vitro*. Reactions (100μl) were assembled using wild-type and Prp8-depleted extracts, incubated for 30 minutes at RT, and precipitated with anti-Prp8 or anti-Prp43p antibodies. Indeed, Prp8-depleted extract does not possess the splicing activity. Additionally, wild-type Prp43p is very weakly associated with spliceosomes under normal conditions as the observed precipitation of splicing products is very close to background levels. On the other hand, Prp43p precipitates an increased amount of pre-mRNA from Prp8-depleted extract, which might indicate the association of Prp43p with early spliceosomal complexes. Precipitation of pre-mRNA with anti-Prp8 antibodies from Prp8-depleted extract is a background precipitation commonly observed in this type of experiment.
Figure 5.2. Depletion of Prp8p has a splicing defect and causes an increase in Prp43p association with pre-mRNA. Splicing reactions (100μl) with normal and Prp8-depleted extracts were assembled. Reactions were incubated for 30 min at RT, products of in vitro splicing reaction were co-precipitated with either anti-Prp8 or anti-Prp43 antibodies (prepared as described). After the precipitation beads were washed extensively, retained RNA was extracted, and resolved in a 7%(w/v) polyacrylamide gel. The gel was dried at 80°C for 2 hours, and subjected to autoradiography.

In order to confirm this result, biotinylated pre-mRNA was used as a substrate for an in vitro splicing assay. Using biotin labelled substrate allows affinity selection of complexes formed on the pre-mRNA and monitoring of protein and snRNAs associations with pre-mRNA. Biotinylated pre-mRNA (bRNA) was generated using in vitro transcription as described in 2.12.2, except that in the nucleotide mixture, modified nucleotide was used in ratio 1 biotin-16-uracil :8 uracil, according to the manufacturer’s (Fermentas) instructions.

To assay snRNAs and protein association with bRNA under splicing conditions, spicing reactions (200μl) were assembled, with non-labelled substrate used as a negative control. After 30 min of incubation at RT, bRNA and associated complexes were precipitated using magnetic streptavidin beads. The magnetic beads are essential for this experiment, as high background was observed with streptavidin.
agarose beads. For snRNA analysis, RNA was extracted from the beads, loaded onto the PAGE gel, blotted and probed for the presence of various snRNAs. For protein analysis, beads were boiled in 2XSDS loading buffer, loaded on 4-12% PAGE gel, blotted and probed for the precipitation of Prp43p and Prp8p. Results are presented in figure 5.3.
Figure 5.3 Prp43p is associated with pre-mRNA in Prp8-depleted extracts. Splicing reactions (200 μl) were assembled with unlabeled substrate or biotinylated RNA, incubated for 30 min at RT, mixed with streptavidin magnetic beads, incubated for 1 hour at 4°C and washed extensively with NTN buffer. For analysis of protein association (panel A), beads were resuspended in 2xSDS loading buffer, and processed as normal protein samples, loaded on a gel 4-12% page gel, blotted, and probed with anti-Prp43 and anti-Prp8 antibodies. For analysis of snRNAs (panel B), RNA was extracted from the beads, and subjected to northern blot analysis as described in 2.12.6.

As shown in figure 5.3B, bRNA efficiently precipitates U2, U1, U5 and U6 snRNAs from a normal splicing extract, suggesting that spliceosomal complexes are assembled on bRNA substrate under these conditions. In the case of Prp8-depleted extract, bRNA precipitates U2 and U1 snRNAs, supporting the fact that depletion of Prp8p causes a defect in spliceosome assembly and causes formation of Complex A under splicing conditions. Low levels of U4, U5 and U6 snRNAs associated with bRNA indicate a residual Complex B complex formation.
Analysis of the protein content of the bRNA precipitates (figure 5.3A) revealed the presence of a band corresponding to Prp8p only in the case of normal (non-depleted) extract, confirming a genuine depletion of Prp8p from the treated extract. At the same time, Prp43p was observed in bRNA precipitates with both normal and Prp8-depleted extracts, but not in the case of unlabelled RNA, indicating that Prp43p association with bRNA is specific and eliminating the possibility that Prp43p association with RNA is due to non-specific binding often described for proteins participating in nucleic acid metabolism. These results taken together suggest that Prp43p is likely to be associated with pre-catalytic spliceosomes as early as Complex A, possibly as a part of U2 snRNP. However, it remains unclear whether Prp43p has a function in early spliceosomes or if it joins the spliceosome as a component of U2 snRNP, and is activated only in the post-splicing complex.
5. 3 Role of the C-terminus of Prp43p in splicing and pre-rRNA processing

It was proposed that the flanking regions determine specific protein-protein, or protein – RNA interactions and possibly regulate the activity of DExD/H box proteins. It was shown that the C-terminus of Prp43p is not required for ATPase activity of Prp43p in vitro, but is essential for cell viability (Tanaka et al., 2007), therefore it was suggested that the C-terminus of Prp43p is required for the protein-protein interactions, or binding of Prp43p to the complexes.

Several temperature-sensitive C-terminus mutants of Prp43p were isolated by our collaborator on this project, Dr G. Edwalds-Gilbert. One of these mutant alleles, prp43E716A, was chosen to assay effect of the C-terminus mutation in vivo on splicing and pre-rRNA processing.

When the work on this project was in progress, it was established that the E716A substitution lies in the region of interaction of Prp43p with Spp382p (Prp43 aa712-720; Tanaka et al., 2007), and therefore this mutant protein is likely to be defective in its interaction with Spp382p. Strikingly, Spp382p also binds ATPase domain of Prp43p, and this interaction is enough to stimulate ATPase activity of Prp43p in vitro, and therefore the role of interaction between Spp382p and the C-terminus of Prp43p in unclear.

In the first instance, growth of the mutant strain at the restrictive temperature was assessed. The mutant strain and isogenic wild type were pre-grown at 30°C, shifted to 37°C, and optical density was measured every two hours. Strain E716A shows slow growth 4 hours after shift to the non-permissive temperature (figure 5.4).
Figure 5.4. Prp43E716A has a defective growth at 37°C. Strain Prp43E716A carrying mutation in the C-terminus and the isogenic wild-type strain were pre-grown at 30°C, and shifted to 37°C. Aliquots of the culture were taken each two hours and their optical density measured.

In order to investigate a possible splicing defect in the mutant strain after the temperature shift, qPCR analysis of splicing products and intermediates was performed. Total RNA was extracted from the Prp43E716A and isogenic wild-type cultures after 4 hours at 37°C, and used for production of cDNA of ACT1 RNA. Several qPCR reactions were set up using sets of oligos (as presented in figure 2.1 in materials and methods, section 2.14) to amplify different RNA species. Relative amounts of different ACT1 RNA species from the mutant strain were compared to those of the wild type (calculated using ΔCₜₜₜ of the PCR reactions performed with cDNA as described in materials and methods section 2.14). As can be seen from the results shown in figure 5.5A, the E716A mutation causes an increase in the level of both lariat and pre-mRNA species, which agrees with the previously described role of Prp43p in spliceosome disassembly and recycling. At the same time levels of mRNA and the level of total RNA (as indicated by the level of the second exon) are
not affected, suggesting that E716A mutation has a mild defect in splicing, which cannot account for the slow growth of the mutant strain at this time-point. To eliminate the possibility that 4 hours at restrictive temperature was not enough for the development of the defect, samples from cultures shifted for 8 hours to non-permissive temperature were prepared. qPCR analysis of mutant sample shows no increase of accumulation of the splicing intermediates in comparison to the earlier time-point (figure 5.5B). A decrease in the accumulation of the splicing intermediates and amount of total ACT1 RNA produced suggests that cell metabolism is severely affected at this time-point, and reporter RNA is not made. Mild accumulation of splicing precursors in the case of the mutant can be explained if this mutant affects late steps of spliceosome disassembly and snRNP dynamics, or the alterRNAtive explanation - this mutant is defective in a process different from splicing.

To assay pre-rRNA processing, northern blot analysis of steady state levels of pre-rRNA precursors following 4 hours shift was performed (Figure 5.5C). No defect in pre-rRNA processing is observed at this timepoint, suggesting that pre-rRNA processing is likely not affected by mutation in the C-terminus of Prp43p.
Figure 5.5 The C-terminus mutant of Prp43p has a mild splicing defect and no defect in pre-rRNA processing after temperature shift. Total RNA extracted from Prp43E716A and BMA38a strains following 4 hours (A and C), and 8 hours (B) shifts were subjected to qPCR analysis and northern blot.
5.4 Prp43 is necessary for U6 snRNA release from the post-splicing complex.

Up to now, little information about snRNP composition of the post-splicing complex is available, and the pathway of spliceosome disassembly is not characterised. As Prp43p plays a role in spliceosome disassembly, the question we wanted to address was whether Prp43p played an early role in spliceosome disassembly, or had a function in partially disassembled spliceosomes. Prp43p was shown to be weakly associated with U2, U5 and U6 snRNAs (Lebaron et al., 2005, Leeds et al., 2006. Combs et al., 2006), which are believed to compose the post-splicing complex. As the ATPase deficient mutant Prp43T123A was shown to stall spliceosomes in excised intron-containing post-splicing complexes (Martin et al. 2002), assaying the snRNP composition of this complex in comparison to wild type would allow us to distinguish between two distinct possibilities and roles of Prp43p in spliceosome disassembly. First, if Prp43T123A is stalled in a genuine post-splicing complex which is formed immediately after mRNA release, in this case the increased association of Prp43T123Ap with U2, U5 and U6 snRNAs is expected, and would suggest that Prp43p acts early in spliceosome disassembly. Another possibility is that Prp43p functions later, and using the mutant form of the protein would lead to accumulation of aberrant complexes limiting the disassembly process.

Two Prp43p mutants - ATPase deficient Prp43T123A and C-terminus mutant Prp43E716A were assayed on their precipitation of snRNAs. The plasmid p113 expressing PRP43T123A from PGAL1 promoter was a gift from Dr. B. Schwer. Strain BMA38a was transformed with the plasmid and transformants were selected on -Trp media. For the extract preparation the plasmid-containing strain was grown in -Trp for pre-culture, and then transferred into -Trp, Gal medium for 8 hours. Prp43T123A has a strong dominant negative effect; growth of the culture is severely reduced with doubling time of more than 8 hours (doubling time of wild-type culture under these conditions is close to 4 hours), which indicates that expression of the dominant negative protein causes effect on the growth.
Splicing extracts were prepared from strains BMA38a and Prp43T123A incubated in Gal medium for 8 hours, and from Prp43E716A shifted for 4 hours to 37°C, and used for immunoprecipitation with anti-Prp43 antibodies. Results are presented in figure 5.7.

![Splicing extracts](image)

Figure 5.7 Prp43 mutant proteins are stalled within spliceosomes. Splicing extracts (100μl) were mixed with 100μl of 1xIPP buffer and protein A Sepharose beads with pre-bound anti-Prp43p antibodies. After immunoprecipituation, RNA was extracted from the beads, and analysed by northern blot.

As can be seen in the figure 5.7 wild-type Prp43p precipitated U2, U1 U5 and U6 snRNAs. Precipitation of U2, U5 and U6 by Prp43p has been previously reported as Prp43p was suggested to function in spliceosome disassembly (Lebaron et al., 2005, Leeds et al., 2006. Combs et al., 2006). Precipitation of U1 snRNA is not surprising as I showed that Prp43p is likely to be associated with the splicesomes as early as Complex A. Previous lack of detected association of Prp43p with U1 can be explained by the fact that all these studies used C-terminal TAP-tagged Prp43p,
while polyclonal anti-Prp43 antibodies were used for my experiments. It is conceivable that in early spliceosomal complexes the TAP-tag on Prp43p is not accessible or the TAP-tagged Prp43p actually has a splicing defect, and presumably affects spliceosome disassembly.

Interestingly, there is a difference in snRNAs precipitation in the case of the prp43 mutants – there is an increased association of Prp43p with U6 and U1 snRNAs and there is a slight increase in the level of the precipitation of U4 snRNA. This not only proves that Prp43p is associated with the spliceosome during all the stages of splicing reaction, but also suggests that spliceosome dynamics in mutants are impaired. Additionally, increased precipitation of U6 by mutant proteins suggests that Prp43p might play a role in U2/U6 duplex unwinding. Moreover, as both ATPase deficient and C-terminal mutants of Prp43p have the same phenotype, it might indicate that the C-terminus of Prp43p plays a role in regulation of ATPase activity of Prp43p in splicing.
5.4 Discussion

In this chapter I show that Prp43p is likely to be associated with the early spliceosomal complexes. I show that Prp43p is present in Complex A using extract unable to form functional Complex B, supporting that Prp43p is present in pre-catalytic spliceosomes. This finding is not surprising as the functional mouse orthologue of Prp43p co-precipitates U2 and U1 under splicing conditions (Gee et al., 1997), suggesting that mPrp43p is associated predominantly with Complex A, and human orthologue of Prp43p is U2 snRNA associated protein (Dybkov et al., 2006). These data indicate that yeast Prp43p is likely to be associated with the spliceosomes during all stages of splicing. It remains to be determined, however, whether Prp43p performs any function early in the spliceosome, and whether this function requires its ATPase activity. To answer these questions additional experiments are required.

Effects of C-terminus mutations in Prp43p on splicing and pre-rRNA processing were investigated. qPCR analysis of splicing intermediates following 4 hours shift to non-permissive temperature revealed the presence of a mild splicing defect with the accumulation of the lariat species and pre-mRNA of the ACT1 gene, which might indicate the presence of the defect in recycling of spliceosomal components in C-terminus mutants. The phenomenon, that prp43 mutants do not show significant accumulation of splicing precursors has been previously reported by J. Combs and colleagues. In their microarray study of ATPase-deficient mutant of Prp43p no drastic changes in levels of splicing precursors and intermediates were observed, and splicing defect of Prp43p mutant was comparable to the splicing defect caused by non-essential splicing factors, which led to proposition that Prp43p participates in pre-mRNA splicing, but has an essential function in pre-rRNA processing. Remarkably, no effect on pre-rRNA processing was noticed for lethal Prp43E716A mutant after the temperature shift. Lack of the effect of the C-terminus mutation on pre-rRNA processing suggests that E716A mutation affects predominantly splicing. The absence of a drastic defect in splicing and pre-rRNA processing, which could explain the growth defect, indicates that the assays used here are not sensitive
enough (or not general enough, e.g. splicing microarray to investigate splicing genome-wide) to reveal the presence of the defect. Interestingly, it was reported that essential splicing factors involved in early stages of splicing do not cause significant accumulation of precursors probably because non-processed precursors are quickly degraded (Combs et al., 2006). It is also possible that similar phenotype will be observed for factors involved in snRNP recycling, and might explain lethality of E716A mutation in the absence of drastic splicing defect.

Another possibility is that C-terminus mutant of Prp43 has an essential function in a process different from splicing and pre-rRNA processing. Interestingly enough, Prp43p was co-isolated with subunits of PolI and PolIII machineries (Lebaron et al., 2005), however the role of Prp43p in transcription has not been investigated yet. This suggests that the C-terminus mutants of Prp43p might have a role in transcription, or coordination of spliceosomal and transcriptional events, but this hypothesis requires further investigation.

It was previously suggested that Prp43p association with spliceosomes is mediated via its C-terminus (Tanaka et al. 2007). As Prp43E716Ap is stalled with snRNAs, it indicates that the C-terminus is not required for Prp43p association with the spliceosome, but rather required for the ATPase activity of Prp43p in splicing. Indeed, a role of the C-terminus in regulation of activity of several DExD/H box helicases was reported (reviewed in Silverman et al., 2003). The way this regulation occurs, however, remains unknown.

Analysis of snRNAs associated with mutants of Prp43p shows an accumulation of U2, U1 and most noticeably, U6 snRNAs. Lack of the accumulation of U5 snRNA might indicate that U5 snRNA is not tightly associated with U2/U6 duplex, and likely to be the first one to leave the post-splicing complex. It is proposed that the main role of U5 snRNP in splicing is exon alignment (Newman and Norman, 1992), and after release of spliced mRNA, U5 snRNA has no RNA-RNA interactions within the post-splicing complex. Therefore, it is likely that U5 snRNA is probably weakly associated with the rest of the components of the post-spliceosome. On the other hand, accumulation of U6 snRNA with Prp43p mutants indicates that ATPase
activity of Prp43p might be required for the release of U6 snRNA from the post-splicing complex. It is not entirely clear whether Prp43 plays a role in unwinding of U2/U6 duplex, or it serves as an accessory factor for another helicase. Processive DEIH helicase Brr2p was shown to play a role in spliceosome disassembly (Small et al., 2006), and was proposed to unwind U2/U6 duplex. Additionally, Brr2p was shown to unwind U2/U6 duplex in vitro (Xu et al., 1996). This allows us to propose that Prp43p might play a role in activation of Brr2p and thus facilitates U2/U6 duplex unwinding, and spliceosome disassembly. Previously role of conformational switches between splicing reactions was proposed for DEAR-box proteins Prp16p and Prp22p (Query and Konarska, 2005). It is conceivable that Prp43p plays a role of such a switch in the post-splicing complex, which initiates spliceosome disassembly. Therefore, the precise nature of RNA-RNA or RNA-proteins rearrangements performed by Prp43p during spliceosome disassembly remains to be identified.
Chapter 6

**Prp43p requires Spp382p for pre-rRNA processing.**

### 6.1 Introduction

Prp43p and Spp382p are implicated in both splicing and pre-rRNA processing. In splicing Prp43p and Spp382 play a role in spliceosome disassembly (Martin et al., 2003; this thesis), while the role of these proteins in pre-rRNA processing remains to be identified. In the splicing process multiple reports showed that Spp382p is a likely accessory factor for Prp43p. It was established that Spp382p is not only required for Prp43p association with the post-splicing complex (Tsai et al., 2005; Boon et al., 2006; this thesis), but also stimulates ATPase activity of Prp43p *in vitro* (Tanaka et al., 2007).

In this chapter I present evidence that Spp382p is required for the ATPase activity of Prp43p in pre-rRNA processing. I also show that the G-patch motif of Spp382p rescues a defect caused by Spp382p-depletion at 30°C, but has a ts phenotype at 37°C. Further investigation of the cause of the decreased viability at elevated temperature shows that a G-patch reconstituted strain has a defect in splicing, not pre-rRNA processing. Additionally, over-expression of Spp382p affects culture growth and causes delocalisation of Prp43p from nucleolus to nucleoplasm and cytoplasm.
6.2 Spp382p is an accessory factor for Prp43p in pre-rRNA processing.
As it was established that Spp382p physically interacts with the intermediates of pre-rRNA processing, and repeats the pattern of precipitation of Prp43p, it was suggested that Spp382p might act as an accessory factor for Prp43p in the pre-rRNA processing machinery as well as in splicing.

In splicing, Spp382p is required for the stable association of Prp43p with the post-splicing complex, and as Spp382p and Prp43p are likely to act together in pre-rRNA processing, it was investigated whether the depletion of Spp382p affects Prp43p association with the pre-rRNA particles. For the experiment strain BK4H (with SPP382 under control of P GAL1 promoter) and BMA38a (isogenic wild type) were used.

BMA38a and BK4H strains were pre-grown in YPGR, aliquots of the culture were inoculated into pre-warmed fresh YPDA medium thus in 4 hours cell cultures will have OD₆₀₀ 0.6-0.8. Extracts from 2 litres of cultures of BMA38a and BK4H were prepared and used for co-immunoprecipitation with anti-Prp43 antibodies. Results are shown in figure 6.1.
Figure 6.1. In the absence of Spp382p, Prp43p is stalled with pre-rRNA particles. Extracts were prepared from 2l of exponentially grown cultures of BMA38a and BK4H after transfer to YPDA for 4 hours for depletion of Spp382p. Extracts were mixed with Protein A Sepharose beads with bound anti-Prp43 antibodies, and incubated for 1 hour at 4°C. After precipitation, beads were washed with lysis buffer and subjected to RNA extraction. For Total RNA samples RNA corresponding to 1% of the amount of the extract used for co-immunoprecipitation was extracted. RNA samples were separated on agarose and PAGE (6%) gels, blotted and probed for the presence of the various pre-rRNA species.

Despite an obvious pre-rRNA processing defect in Spp382p-depleted extract, the increased precipitation of Prp43p with the pre-rRNA precursors cannot be explained solely by the increased abundance of the particular intermediate. Thus, depletion of Spp382p causes an accumulation of Prp43p with 35S, aberrant 23S, 20S, U3 snoRNA (levels of precipitation of these species by mutant strain was on average 5 times more than their level of precipitation in wild-type strain, and close to 1%). No
significant changes in precipitation of 27SB and 7S species were observed. Only a very small proportion of mature species was precipitated (less than 0.2%), which is likely to be insignificant. This result suggests that in the absence of Spp382p, Prp43p is stalled with pre-rRNA processing machinery, and this is especially pronounced for small subunit pathway. Interestingly, it was reported that ATPase deficient mutants of Prp43p accumulate with pre-rRNA processing particles (Lebaron et al., 2005), therefore increased association of Prp43p with pre-rRNA species in Spp382-depleted extract indicates that Spp382p might affect ATPase activity of Prp43p in pre-rRNA processing. This finding strongly suggests that Spp382p is an accessory factor for Prp43p in pre-rRNA processing as well as in splicing.

U3 snoRNA is required for the cleavages at A₀, A₁ and A₂ sites and directly interacts with pre-rRNA. As Spp382-depletion primarily affects A₀, A₁ and A₂ cleavages, therefore accumulation of Prp43p with U3 snoRNA reflects accumulation of Prp43p with 90S particles. Interestingly, Prp43p also precipitates unspliced pre-U3 snoRNA from extract depleted of Spp382p supporting the idea that Prp43p is likely to have an earlier function in the spliceosome, and that progression of spliceosome assembly is impaired in Spp382-depleted extract.

6.3 G-patch of Spp382p rescues growth defect caused by depletion of Spp382p.

A recent report by Tanaka et al., showed that the G-patch of Spp382p alone was able to stimulate the ATPase activity of Prp43p in vitro on a level similar to that of the full-length protein (Tanaka et al., 2007). We therefore investigated whether G-patch alone would be enough to support viability of Spp382-depletion in vivo.

In order to test this, the G-patch of Spp382 (aa 1-120) was cloned under control of the SPP382 promoter in the XhoI site of the pRS316 vector to produce pTAG-1. Yeast strains BMA38a and BK4H were transformed with pTAG-1, and selected on Ura medium with a mixture of 2% raffinose and 2% sucrose as carbon source, as no transformants were obtained in galactose-containing medium for BK4H strain at
30°C. One possible explanation is that over-expression of SPP382 from \( P_{GAL1} \) and the G-patch from the native promoter is toxic for the cells. Indeed, it was reported that over-expression of SPP382 causes decreased viability (Herrmann et al., 2007), and therefore control and G-patch reconstituted strains were grown in 0.5% Gal, 2% Raf and 2% Suc (GRS) in order to minimize the over-expression of SPP382. Viability of the strains was tested on UraGR medium. BMA38a, BK4H and G-patch reconstitution strains (BK4H+pTAG-1) were pre-grown in GRS medium, spotted on GR agar and YPDA agar, and incubated at 30°C or 37°C for 3 days. As can be seen in figure 6.2, panel A, BK4H shows slight delay in growth in comparison with the wild type, while the G-patch reconstitution strain grows slower than the BK4H strain. This suggests that the Spp382p and G-patch dosage might have an effect on growth of yeast cells. Interestingly, the result presented in figure 6.2, panel B indicates that G-patch alone was enough to support viability of the BK4H strain at 30°C in YPDA as G-patch reconstituted strain grows in YPDA medium with a rate similar to the wild-type strain. In contrast, strain BK4H does not grow on YPDA medium, showing that depletion of Spp382p severely affects cell growth (several colonies were observed for BK4H strain after 7 days of incubation in YPDA, therefore the possibility of very slow growth cannot be excluded). As growing G-patch reconstitution strain at 30°C in GR medium affected cell growth, all subsequent experiments with the G-patch reconstitution strain were performed using YPDA or Ura medium. In figure 6.2, panel C is shown that growth of the G-patch reconstituted strain is affected by elevated temperature, therefore G-patch reconstitution strain has a \( ts \) phenotype.
Figure 6.2. G-patch of Spp382p rescues growth the defect caused by depletion of Spp382p. Spot assay with wild-type strain with pRS316 and BK4H (SPP382 under \( P_{GALI} \) promoter control) with pTAG-1 (containing G-patch of Spp382p expressed from native SPP382 promoter in pRS316 vector). Cultures were pre-grown in Ura medium. 0.3 OD\( _{600} \) of the each culture was spun, washed and resuspended in 50\( \mu l \) of water. Serial 5x dilutions were set up, and spotted on the plates. Plates were incubated for 3 days at 30 or 37°C.

As Spp382p participates in both splicing and pre-rRNA processing, the growth defect of G-patch reconstitution at 37°C might be determined by aberrant splicing or pre-rRNA processing, or both. To investigate the defect further, total RNA was extracted from the cultures of G-patch reconstitution strain and wild-type strain shifted to 37°C for 8 hours. No defect in growth-rate was observed at this time, however it was noticed that after the temperature shift most of the mutant cells increased in size with enlarged vacuoles, which might alter the optical density.
readings. Splicing specific qPCR analysis of ASC1 transcripts revealed the presence of a mild splicing defect with the accumulation of the lariat species and pre-mRNA, which indicates a snRNP recycling defect in the G-patch reconstituted strain at elevated temperature (figure 6.3, Panel A). Spp382p is an accessory for Prp43p in spliceosome disassembly, therefore it is possible that in the G-patch reconstituted strain at 37°C the function of Prp43p is impaired. Association of snRNAs with Prp8p and Prp43p was investigated in extract prepared from G-patch reconstituted strain shifted to 37°C for 8 hours. For control sample, splicing extract was prepared from a culture of BMA38a strain treated in the same way. 100μl of each extract were mixed with beads with pre-bound anti-Prp8 and anti-Prp43 antibodies. After immunoprecipitation retained RNA was extracted from the beads, resolved in a gel, blotted and hybridised with probes to snRNAs. The result is presented in figure 6.3, Panel B.
Figure 6.3. G-patch reconstitution strain shows a splicing defect at non-permissive temperature.
Panel A. *ASCI* splicing was assayed by qPCR analysis. Relative abundance of RNA species in G-patch reconstituted strain is shown.
Panel B. Co-precipitation of snRNAs with Prp8p and Prp43p from wild-type and G-patch reconstituted extracts after 8 hours temperature shift. Splicing extracts (100μl) were mixed with an equal volume of 2XIPP buffer containing beads with pre-bound anti-Prp8 and anti-Prp43 antibodies. Samples were incubated for 1 hour at 4°C, RNA was extracted from the beads and resolved on the 6% PAGE gel, blotted and probed with specific snRNA probes.

Strikingly, at least two times increase in amount of U6 snRNA associated with Prp43p in G-patch reconstituted extract was repeatedly observed, which suggests that snRNA dynamics in G-patch-reconstituted strain is impaired. Interestingly, increased precipitation of U6 was observed for C-terminus and ATPase deficient mutants of Prp43p (previous chapter), which suggests, that in the G-patch reconstituted strain the function of Prp43p in splicing is impaired.

In order to investigate whether G-patch reconstitution causes a defect in pre-rRNA processing, total RNA extracted from the cultures incubated at 37°C was subjected to
northern blot analysis. As Spp382-depletion primarily affects the levels of 35S and 20S pre-rRNAs, these species were investigated as a hallmark of the Spp382-depletion defect in pre-rRNA processing. Total RNA samples (2mg each) were resolved on denaturing agarose gel, and probed for accumulation of 35S and 23S, and decrease of 20S. However no accumulation of pre-rRNA processing intermediates was observed in the reconstituted strain (figure 6.4 A), suggesting that G-patch reconstitution rescues the growth defect caused by Spp382-depletion in pre-rRNA processing. There is a possibility that lack of the accumulation of the pre-RNA precursors is due to the low expression of pre-rRNA. Therefore in order to fully eliminate the presence of the pre-rRNA processing defect, pulse chase analysis of rRNA processing was performed.

For the pulse chase experiment both BMA38a (with pRS316 plasmid) and yTAG-1 strains were pre-grown at 30°C in -Ura, and then transferred to 37°C for 8 hours. Pulse (1 min) was performed using \(^{3}\)H-Uracil, then excess of non-labelled Uracil was added, and aliquots were taken at 0, 2.5, 5, 10 and 20 minutes. Total RNA was extracted, resolved on denaturing agarose gel and blotted. Membrane was dried and used for autoradiography.
Figure 6.4. G-patch reconstituted strain does not have a defect in pre-rRNA processing.
A. Total RNA from wild-type and G-patch reconstitution strains was extracted following 8 hours temperature shift, resolved on the gel, and blotted.
Panel B. Pulse chase analysis of G-patch reconstitution after 8 hour shift to 37°C. RNA was labelled with $^3$H-uracil for 1 min and chased with an excess of cold uracil. Samples were collected at the indicated time-points, Total RNA was extracted and resolved on a 1.2% agarose gel, and then transferred to HybondN+ membrane. The membrane was subsequently subjected to autoradiography.

As can be seen in figure 6.4 panel B, the G-patch reconstitution strain shows the same kinetics of pre-rRNA processing as the wild-type strain, which indicates that there is no kinetic defect in pre-rRNA processing between G-patch reconstituted strain and wild type after 8 hours of incubation at 37°C. These results indicate that
the growth defect of yTAG-1 strain at elevated temperature is caused by aberrant splicing, not aberrant pre-rRNA processing.

6.4 Over-expression of Spp382p causes delocalisation of Prp43p.
As previously stated, Prp43p and Spp382p are shared between the splicing and pre-rRNA processing machineries. Pre-rRNA processing largely takes place in the nucleolus. Only late stages of 60S maturation occur in the nucleus, and late stages of 40S maturation occur in the cytoplasm. Prp43p is predominantly nucleolar (Combs et al., 2006), consistent with its function in pre-rRNA processing, and it was also found to be associated with late 60S and 40S precursors, suggesting that there is a small sub-population of Prp43p, which is located in nucleoplasm and cytoplasm. Additionally, splicing takes place in the nucleoplasm, and this suggests that there is a nuclear sub-population of Prp43p involved in splicing. As Prp43p is connected to the complexes found in nucleolus, nucleoplasm and cytoplasm, it is likely that Prp43p shuttles between the two processes, however evidence of the shuttling was missing.

Immuno-fluorescent staining was used to analyse localisation of Prp43p in BMA38a and in a strain over-expressing Spp382p. For this, BK4H and BMA38a strains were grown in YPGR, and used for immuno-fluorescent staining analysis. In wild-type cells Prp43p is localized to the nucleolus (as indicated in figure 6.5, Panel A) consistently with the previous report (Combs et al., 2006). Surprisingly, over-expression of Spp382p causes delocalisation of Prp43p from the nucleolus to nucleus and cytoplasm (figure 6.5, Panel B), suggesting that Prp43p shuttles between cellular compartments and processes. It was observed that over-expression of the proteins might promote accumulation of the proteins in non-functional complexes, or aggregate formation. Therefore, it is not clear whether Prp43p forms aggregates with Spp382p, or it accumulates in aberrant high-molecular weight complex(es). Preliminary data (not shown) indicate that Prp43p is likely to be stalled in aberrant high molecular weight complexes.
Figure 6.5. Over-expression of Spp382p causes delocalisation of Prp43p. Immuno-fluorescent staining of a strain over-expressing SPP382 was performed. Affinity purified rabbit anti-Prp43p antibodies and mouse anti-Nop1 antibodies were used for immunostaining in dilution 1:500. Secondary antibodies goat anti-mouse AlexaFluor 488 and goat anti-rabbit AlexaFluor594 were used for the visualisation of the proteins.
6.4 Discussion

In this chapter I show a role of Spp382p as an accessory factor for Prp43p in pre-rRNA processing. I demonstrate that in the absence of Spp382p, Prp43p accumulates with pre-rRNA processing particles. There are several plausible explanations for this. First, lack of Spp382p might result in loss of Prp43p’s ATP hydrolysis/helicase activity and therefore results in stable association of Prp43p with the RNPs. This is supported by the observation that ATPase deficient mutants of Prp43p are stalled in post-splicing complex and in pre-RNA processing particles (Martin et al., 2003, Lebaron et al., 2005). Another possibility is that the presence of Spp382p is required for the release of Prp43p from the RNPs after ATP hydrolysis. In this case Spp382p may provide a binding site for an unknown release factor for Prp43p, or Spp382p might play the role of the release factor itself. However, as Spp382p was already shown to stimulate helicase activity of Prp43p directly, it is more likely that Spp382p is required for the helicase activity of Prp43p in pre-rRNA processing than Spp382p plays two independent and different roles in regulation of the activity of Prp43p. Interestingly, Prp43p does not require Spp382p for association with the pre-rRNA particles, while in splicing it is required for the stable association of Prp43p with the post-splicing complex. This implies that Prp43p-Spp382p dimer might form a complex with dual properties and functions.

In this chapter I also show that the G-patch of Spp382p alone is able to reconstitute viability of otherwise lethal Spp382-depletion at 30°C. It is not entirely surprising as G-patch alone stimulates the helicase activity of Prp43p in vitro on a level similar to that of full-length Spp382p (Tanaka et al., 2007). Further investigation revealed that the G-patch reconstitution strain has a mild ts phenotype with decreased growth at 37°C. qPCR analysis of RNA extracted from the G-patch reconstitution strain following a shift to 37°C for 8 hours shows accumulation of pre-mRNA and excised intron-lariat, suggesting a recycling defect. This result was confirmed by the report from Tanaka et al., that G-patch alone cannot substitute for full-length Spp382p in vitro splicing reaction (Tanaka et al., 2007). Co-immunoprecipitation of Prp8p and Prp43p with snRNAs revealed an increased association of U6 snRNA with both
proteins, as in case with ATPase deficient and C-terminal mutants of Prp43p, suggesting that in the G-patch reconstitution strain elevated temperature affects Prp43p function in spliceosomes. Surprisingly, the reconstitution strain does not show any defect in pre-rRNA processing at the time-point tested, which indicates that the ts phenotype of G-patch reconstitution is due to the defect in splicing, not pre-rRNA processing.

Delocalisation of Prp43p in the strain over-expressing Spp382p supports the idea that Prp43p shuttles between spliceosomal and pre-rRNA processing machineries. Despite it is not entirely clear whether Prp43p forms aggregates with Spp382p, or accumulates in large molecular complexes, I favour the latter hypothesis. Overexpression of Spp382p affects cell viability (this thesis, Herrmann et al., 2007), suggesting that a dosage effect of Spp382p is important. It is possible that decreased cell viability is caused by partial displacement of Prp43p from its functional sites. Indeed, a mild defect in pre-rRNA processing was observed (data not shown) in the strain over-expressing SPP382, suggesting that Spp382p titrates away or saturates binding sites of the components essential for pre-rRNA processing. As the biological significance of sharing components between two processing machineries is not known, the existence of the gene-dosage effect indirectly supports the idea of the role of Prp43p and Spp382p in a concerting mechanism.
Chapter 7

7 Final discussion and future experiments

7.1 The role of Prp43p in splicing
DEAH-box helicase Prp43p is implicated in both splicing and pre-rRNA processing. ATPase mutants of Prp43p are stalled with the multiple pre-rRNA processing particles and post-splicing complex. Remarkably, C-terminus mutant of Prp43E716Ap affects splicing, but not pre-rRNA processing. Isolation of the mutant of Prp43p with predominant defect in splicing will allow identifying the target of the helicase activity of Prp43p in the post-splicing complex. As \textit{prp43} mutants affect snRNP dynamics, and cause accumulation of U2/U6 duplexes, it is likely that the activity of Prp43p affects U2/U6 dissociation, however, cross-linking of the C-terminus mutant of Prp43p to RNA is required to support this hypothesis. Additionally, as no post-splicing complex was isolated, using this mutant would allow investigation of the protein content of the post-splicing complex.

DEIH helicase Brr2p was suggested to unwind U2/U6 duplex in post-splicing complex, and as \textit{prp43} mutants cause an accumulation of aberrant RNP complexes, it suggests that Prp43p might govern Brr2p activity. Brr2p is a only ATPase of the post-splicing complex, and U2/U6 unwinding is believed to be strictly ATP-dependent, suggesting that Brr2p unwinds U2/U6 in post-splicing complex. Other factors implicated in regulating Brr2p activity in spliceosome disassembly are Snu114p and Prp8p. So far no direct interaction of Prp43p with Brr2p, Prp8p or Snu114p has been reported, and it remains to be determined how the Prp43p influences Brr2p activity. For better understanding of the regulation events, it is important to investigate interactions between these proteins.

It is shown that decreasing the ATPase activity of Prp43p suppresses the \textit{prp38-1} mutant. The \textit{prp38-1} mutant causes retention of U4 in Complex B probably by affecting Brr2p activity. As the level of the suppression is inversely proportional to the level of residual ATPase activity of Prp43p, it indicates that perhaps decreasing
Prp43p activity gives more time for the remodelling step involving Prp38p and Brr2p to occur, suggesting that Prp43p is involved in modulation of Brr2p activity in spliceosome assembly as well as disassembly.

### 7.2 The role of Prp43 in pre-rRNA processing

Prp43p was co-isolated with multiple pre-rRNA processing particles, however, the role of Prp43p in pre-rRNA processing is not clear, which opens a broad range for future research. Several potential co-factors of Prp43p in pre-rRNA processing have been identified (Lebaron et al., 2005); they are Spp382p, Gno1p and Pfalp. Significantly, they all have a G-patch motif – a putative RNA binding domain, however, the biological role of this is not known. However it was proposed that G-patch motif plays an important function in regulation of activity of DEAH-box proteins (Silverman et al., 2004). Intriguingly, Spp382p, Gno1p and Pfalp have different localization sites in the cell. Thus, Gno1p is nucleolar, Spp382p is nuclear and Pfalp is nuclear and cytoplasmic (Herrmann et al., 2007; Huh et al., 2003), which allows hypothesize that activity of Prp43p can be regulated in site-specific way by these proteins. Additionally, these co-factors might bind same region of Prp43p. At the same time, only sites of interaction of Prp43p with Sp382 were mapped (Tanaka et al., 2007), and nothing is known about whether the binding sites of Pfalp and Gno1p overlap with binding site of Spp382p. Therefore mapping binding sites of Pfalp and Gno1p with Prp43p might be important to establish how the activation of Prp43p occurs.

ATPase deficient mutants of Prp43p are stalled with multiple pre-rRNA precursors, which implies that the ATPase activity of Prp43 is required for several stages of pre-rRNA processing (Lebaron et al., 2005). As DExD/H box proteins might function as fidelity factors, the possibility that Prp43p is a fidelity factor in pre-rRNA processing should be investigated. Identification of potential targets of Prp43p and investigation of effects of ATPase deficient yet viable mutants of Prp43p on pre-rRNA processing might provide a way to uncover role of Prp43p as a fidelity factor.
7.3 Crosstalk
Several factors were identified as shared subunits between pre-rRNA processing and spliceosomes. Those include Spp382p, Prp43p, Snu13p and potentially Pfalp. The significance of this is not known. While sharing of Snu13p can be explained by likely common evolutionary origin between RNAs it interacts with (Watkins et al., 2000), nothing can be stated about Prp43p and Spp382p as their targets have not been identified yet. However, it is likely that Prp43p and Spp382p form a complex, and putative shared protein Pfalp might be a part of this complex based on reported interactions. Thus Pfalp co-purifies with Prp43p (Lebaron et al., 2005), and genetic interaction between Spp382 and Pfal was reported (Pandit et al., 2006). The interaction between Spp382p and Pfalp is based on a gene-dosage effect as over-expression of Pfalp antagonizes the suppression of the prp38-1 mutation by spp382 mutants (Pandit et al., 2006). This suggests that these proteins might compete for the same binding partners, or have overlapping binding sites within Prp43p. Despite genetic interaction of Pfalp with spliceosomal components was reported, it is not known whether Pfalp participates in splicing, and therefore it would be interesting to determine this for the further understanding of subunits sharing.

Normally Prp43p is localised to the nucleolus (Combs et al., 2006). Over-expression of Spp382p causes delocalisation of Prp43p from the nucleolus to the nucleus and the cytoplasm, which suggests that Prp43p is shuttling between RNA processing machineries. It is not clear whether Prp43p is accumulated with Spp382 in a non-functional dimer or Spp382p saturates binding sites for co-factors of Prp43p causing formation of aberrant high molecular weight complexes. These possibilities can be easily distinguished by gradient centrifugation analysis of the strain over-producing Spp382p.

It is established that Prp43p accumulates with the catalytic spliceosomes assembled on aberrant substrates (these thesis, Pandit et al., 2006). Therefore if Prp43p shuttles between processing machineries a defect in splicing might cause depletion of Prp43p from pre-rRNA processing machinery and vice versa. For pre-rRNA processing, U3 snoRNA and many ribosomal protein pre-mRNAs are required to be spliced.
However, U3 snoRNA has a long half-life and mRNAs encoding ribosomal proteins are resistant to splicing defects (Pleiss et al., 2007). This suggests that it is very unlikely that defective splicing will have an immediate effect on pre-rRNA processing. Hypothetically, pre-rRNA processing is dependent on correct splicing and might require a way to identify that splicing is taking place. In this case sharing subunits between machineries might provide a way of concerting ribosomal biogenesis, and additionally it might provide a quick way of slowing down pre-RNA processing machinery before depletion of U3 and r-proteins occurs. Interestingly, Prp43p is also found to be associated with the PolI and PolIII subunits (Lebaron et al., 2005), however a role for Prp43p in transcription has not been identified. Association of Prp43p with multiple RNA processing machineries suggests the hypothesis that its potential role is as a universal chaperon, connecting transcription, splicing and pre-rRNA processing in ribosomal biogenesis.
Bibliography


123


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Yeast Ntr1/Spp382 Mediates Prp43 Function in Postsplicing Complexes

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The Ntr1 and Ntr2 proteins of Saccharomyces cerevisiae have been reported to interact with proteins involved in pre-mRNA splicing, but their roles in the splicing process are unknown. We show here that they associate with a postsplicing complex containing the excised intron and the spliceosomal U2, U5, and U6 snRNAs, supporting a link with the late stage in the pre-mRNA splicing process. Extracts from cells that had been metabolically depleted of Ntr1 has low splicing activity and accumulates the excised intron. Also, the level of U4/U6 di-snRNP is increased but those of the free U5 and U6 snRNPs are decreased in Ntr1-depleted extract, and increased levels of U2 and decreased levels of U4 are found associated with the U5 snRNP protein Prp8. These results suggest a requirement for Ntr1 for turnover of the excised intron complex and recycling of snRNPs. Ntr1 interacts directly or indirectly with the intron release factor Prp43 and is required for its association with the excised intron. We propose that Ntr1 promotes release of excised introns from splicing complexes by acting as a spliceosome receptor or RNA-targeting factor for Prp43, possibly assisted by the Ntr2 protein.

The excision of introns from precursor mRNAs (pre-mRNAs) occurs by two consecutive transesterification reactions in the spliceosome, a large and highly dynamic ribonucleoprotein complex (9). These chemical reactions are likely catalyzed by small nuclear RNAs (snRNAs) that exist within small nuclear ribonucleoprotein particles (snRNPs), but non-snRNP proteins also play essential roles such as conferring specificity, checking the fidelity of the process, and regulating conformational rearrangements in the spliceosome (8, 34, 42). Five snRNPs, called U1, U2, U4, U5, and U6, assemble on the substrate pre-mRNA to form the spliceosome. First, the U1 snRNP binds at the 5′ splice site, followed by the U2 snRNP at the branch point, and then the U4, U5, and U6 snRNPs, in the form of a U4/U6.U5 tri-snRNP, join the assembling complex. Activation of the assembled spliceosome requires dynamic remodeling of an intricate network of RNA-RNA and RNA-protein interactions within the spliceosome such that the U1 and U4 snRNPs are released. Concomitantly, the Prp19-associated complex of proteins (nineteen complex or NTC) (27, 28, 35, 36) associates with the spliceosome, remodeling the U5 snRNP (24, 25) and stabilizing interactions of the U5 and U6 snRNAs with the pre-mRNA (10, 11) prior to the first catalytic step of splicing.

Upon completion of the splicing reaction, the spliced exon RNA (mRNA) is released and the postsplicing ribonucleoprotein complex dissociates in an active process that involves two members of the ATP-dependent DEAH box RNA helicase family, Prp22 and Prp43 (3, 26, 32). Prp22 is needed for release of the spliced exons (32, 43), while Prp43 is required for disassembly of the spliceosome and release of the excised intron in its branched, lariat form (26). The U4 snRNP reassociates with the U6 snRNP (31, 41) to form the U4/U6 di-snRNP that will then join the US snRNP to form the U4/U6,U5 tri-snRNP once more.

Although many protein components of the splicing machinery have been identified through their physical or genetic interactions with known splicing factors, the functions of a large number of these remain to be determined. For example, the name SPP382 (S. Pandit and B. C. Rymond, unpublished results; http://dbyeastgenome.org/cgi-bin/locus.pl?locus=spp382) was given to Saccharomyces cerevisiae open reading frame YLR424W to indicate its ability to suppress the prp38-1 mutation that causes a defect in spliceosome maturation (45); however, the function of the Spp382 protein is unknown. Spp382 has been reported to associate directly or indirectly with many protein components of the spliceosome, including Prp43 and components of the U5 snRNP (1, 7, 13, 16), and recently the name Ntr1 (nineteen complex related) was proposed because of its interaction with the NTC (38). In addition, Spp382/Ntr1 interacts with Ykr022c/Ntr2 (16, 17, 38, 39).

We demonstrate here that Ntr1 and Ntr2 are spliceosome associated and coprecipitate mainly excised intron from an in vitro splicing reaction mixture. Under nonsplicing conditions, Ntr1 and Ntr2 coprecipitate the U2, U5, and U6 snRNAs. Northern and microarray analyses show increased levels of pre-mRNAs in cells depleted of Ntr1, and in vitro, Ntr1-depleted extract has low splicing activity and accumulates excised intron in a postsplicing complex that includes Prp8 and Cef1. Glycerol gradient analysis of Ntr1-depleted extract suggests a defect in recycling snRNPs, and the U5 snRNP protein Prp8 is shown by immunoprecipitation to be associated with eightfold more U2 snRNA and with U6 snRNA that is not complexed with U4 snRNA, supporting its accumulation in the U2/U5/U6 postsplicing complex. The intron release factor Prp43 cofractionates with Ntr1 and Ntr2, and in the absence of Ntr1, Prp43
TABLE 1. Plasmids and yeast strains used in this study

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TABLE 2. Deoxyoligonucleotides used in this study

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Oligonucleotides for C-terminal NTR2 TAP tagging

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Oligonucleotides for PCR amplification of NTRI from genomic DNA (BMA38a) and cloning into pGEX4T-2 via Sall and NotI restriction sites

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Oligonucleotides used for C-terminal NTR1 TAP tagging

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Oligonucleotides for C-terminal NTR1 Pgal1 promoter and triple HA tagging

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is unable to associate with spliceosomes to release the excised intron. These results complement and extend the recently reported results of Tsai et al. (38), demonstrating that Ntrl and Ntr2 function in the Prp43-mediated release of the excised intron. We propose that Ntrl acts as a spliceosome receptor or RNA-targeting factor to promote interaction of Prp43 with the excised intron, possibly assisted by the Ntr2 protein.

MATERIALS AND METHODS

Yeast strains and plasmids. The S. cerevisiae strains and plasmids used in this work are described in Table 1, and the oligonucleotides used are described in Table 2. For metabolic depletion of Ntrl, cultures of KL4G or KL4G2T were grown in YPGR (1% yeast extract, 2% Bacto Peptone, 2% galactose, 2% raffinose) medium to an optical density at 600 nm (OD600) of 0.5, promptly washed with YPD (1% yeast extract, 2% Bacto Peptone, 2% glucose) medium downgrowth, resuspended in the original culture volume of YPD, and grown for 4 h as indicated (with addition of fresh YPD to keep the cells in log phase), and transformation was performed as described previously (14). Strains KL4T, KL4G, and KL4G2T were generated from BMA38a (1) by one-step transformation by using plasmid DNAs as templates (Table 1).

Splicing extract preparation and in vitro splicing analysis. Preparation of whole-cell yeast extracts was performed as described previously (40). Splicing reaction mixtures were prepared as described previously (22). In vitro splicing reaction mixtures contained part of the yeast ACT1 gene, was transcribed in vitro by T7 RNA polymerase and [α-32p]ATP to produce a uniformly labeled substrate (29).

Glycerol gradient analysis. For glycerol gradient analysis (essential for the analysis of splicing extract (without added ATP)), the extracts were centrifuged at 106,000 g for 3 h and one-fifth of the supernatant (closed to the top of the tube) was applied to a 10-40% (w/v) glycerol gradient in 10 mM Tris-HCl (pH 7.4) and run at 100,000 rpm for 24 h in a Beckman SW40.1 rotor (Beckman Instruments, Palo Alto, CA) at 4°C. The gradient was fractionated from the bottom to the top in 1-ml fractions, and the fractions were analyzed by SDS-PAGE. The positions of the initiation and termination events were determined by autoradiography and the positions of spliced products were determined by the addition of a primer containing an EcoRI site at the 3' end.
RESULTS

Ntr1 and Ntr2 proteins associate with a postsplicing intron-containing complex. To investigate whether the Ntr1 or Ntr2 protein associates with spliceosomal snRNPs, we tested the ability of C-terminally tandem affinity purification (TAP)-tagged Ntr1 (Ntr1-TAP) or Ntr2 (Ntr2-TAP) protein to coprecipitate the U1, U2, U4, U5, or U6 snRNA. As shown in Fig. 1A, both Ntr2-TAP and Ntr1-TAP, when immunoprecipitated in the presence of 150 mM salt, coprecipitated the U2, U5 (L and S), and U6 snRNAs but not U1 or U4 snRNA above the background (compare lanes 5 and 6 with lane 4). This indicates that each of these proteins associates with a significant amount of the U2, U5, and U6 snRNPs (approximately 20% of the U2 and U5 snRNPs were precipitated; note that U6 snRNPs is normally present in excess over the other spliceosomal snRNPs). To determine directly whether the Ntr1 or Ntr2 protein interacts with spliceosomes, the association of the TAP-tagged proteins with ACT1 substrate RNA or the intermediate or products of its splicing in vitro was tested by immunoprecipitation. As previously reported (37), the U5 snRNP protein Prp8 coprecipitated unspliced RNA, as well as intermediates and products of the splicing reaction (Fig. 1B, lane 7). In contrast, Ntr1-TAP and Ntr2-TAP coprecipitated the excised intron preferentially over the other RNA species (Fig. 1B, lanes 8 and 9). The U2, U5, and U6 snRNPs have been found in a postsplicing complex that contains the excised intron, following release of the spliced exons in a HeLa cell splicing reaction mixture (19). Thus, the association of the Ntr1 and Ntr2 proteins with U2, U5, and U6 snRNPs and with excised intron RNA most likely indicates their association with a postsplicing intron-containing complex and suggests either that they may be absent at earlier stages of the splicing process or that the TAP tag was masked within earlier spliceosome complexes.

Depletion of Ntr1. The NTR1 gene is essential for cell viability. Therefore, to investigate the requirement for Ntr1 protein for splicing, yeast strain KL4G, in which chromosomal NTR1 is transcribed under the control of the glucose-repressible P_GAL1 promoter, was grown in galactose medium and then shifted to glucose medium. As a control, a second culture was treated identically but was transferred back to galactose medium. Approximately 2 h after the metabolic shift, the growth of the culture slowed considerably and then the cells continued to grow slowly (approximately 4-h doubling time; data not shown). Northern analysis of extracted RNA showed a barely
detectable amount of unspliced pre-U3 RNA 2 h after the shift, with the amount increasing for 6 to 10 h postshift (Fig. 2A, lanes 6 to 10). Microarray analysis showed a mild splicing defect for some transcripts of intron-containing genes 2 h after the shift and a substantial genomewide accumulation of unspliced pre-mRNAs by 10 h postshift (data not shown). Extract from KLAG cells grown for 10 h in glucose contained a barely detectable amount of Ntr1 (Fig. 2B, lane 2) and, when incubated under in vitro splicing conditions with an ACT7/I substrate RNA, produced only a small amount of spliced exon but accumulated a substantial amount of excised intron (Fig. 2B, lane 6) compared to nondepleted extract (Fig. 2C, lane 2). Incubation of the reaction mixtures with anti-Prp8 (lanes 9) or anti-Cef1 (lanes 10 and 11) antibodies showed that Ntr1-depleted extract the excised intron is associated with Prp8 and Cef1, supporting its accumulation in a postsplicing complex. When KLAGT2 (same as KLAG but with TAP-Ntr2) cells were treated similarly by growth in glucose to deplete Ntr1, TAP-Ntr2 coprecipitated a substantial amount of excised intron from a splicing reaction mixture (Fig. 10). Therefore, Ntr2 is able to associate with the excised intron in the absence of Ntr1. The dramatic shift in the amounts of the spliced exon and excised intron in the Ntr1-depleted reaction mixture is most likely a consequence of inhibition of splicing as a result of the retention of essential splicing factors within a postsplicing complex that is likely retained in a postsplicing complex to which the excised intron RNA is protected against degradation (26). This indicates that Ntr1 is required for efficient pre-mRNA splicing and for retention of excised intron and suggests that Ntr1 might play a role in recycling the postspliceosome components for new rounds of splicing.

The distribution of snRNPs in extracts from Ntr1-depleted and nondepleted cells was analyzed by fractionation in preparative gradients, followed by Northern blotting. The densest fractions contained U4/U6.U5 tri-snRNPs and possibly some of the postsplicing complexes (Fig. 3C,B, fractions 1 to 7) and then free U5 snRNPs (fractions 8 to 13), free U6 (fractions 15 to 19). Quantification of these results showed that, compared with nondepleted extract, in Ntr1-depleted extract there was a 50% reduction in the amount of U4 snRNA in the high-density fractions (fractions 1 to 3) and a corresponding increase in the amount of U4 snRNA present in the U4/U6 di-snRNP (Fig. 3B, fractions 9 to 13), whereas the free U5 snRNP (fractions 5 to 9) was essentially absent in Ntr1-depleted extract. There was also a small but reproducible decrease in the level of free U6 snRNA (Fig. 3A, fractions 15 to 19) although this is less obvious, as free U6 snRNA is usually not detected. The dramatic shift of U4 snRNA from high-density fractions to the U4/U6 di-snRNP region of the gradient was accompanied by a reduction in the amount of free U5 and U6 snRNPs, presumably of free U2 snRNP, although this cannot be assessed with these gradients. A decrease in the amount of excised intron from a splicing reaction mixture (Fig. 3B, lane 6) compared to nondepleted extract (Fig. 2B, lane 2) and, when incubated under in vitro splicing conditions with an ACT7/I substrate RNA, produced only a small amount of spliced exon.
Depletion of Ntr1 leads to a redistribution of snRNP complexes. To examine the profile of snRNP distribution, non-Ntr1-depleted (A) and Ntr1-depleted (B) cell extracts (no added ATP or pre-mRNA) were centrifuged through 10 to 30% glycerol gradients and RNAs were extracted from alternate fractions and analyzed by Northern blotting with probes for U1, U2, U4, U5, and U6 snRNAs. The sedimentation of snRNP complexes is indicated. In addition to U4/U6, U5 tri-snRNPs and U5 snRNPs, fractions 1 to 7 may contain some endogenous spliceosome and postsplicing complexes.

(C) Coprecipitation of snRNAs by anti-Prp8 antibodies in extracts from Ntr1-depleted (lanes 4 to 6) or nondepleted (lanes 1 to 3) cells. RNAs were analyzed by fractionation in a 6% polyacrylamide–8 M urea gel and Northern blotting. Preimmune (PI) serum was used as a background control. (D) Same as panel C, but the precipitated snRNAs were analyzed by nondenaturing gel electrophoresis as described previously (41). IP, immunoprecipitation.

(Fig. 3C, lane 6) and an equivalent amount of U6. The increased association of Prp8 with a U2 snRNA-containing complex and decreased association with U4 snRNP in Ntr1-depleted extract supports the accumulation of Prp8 in the U2/U5/U6 excised intron-containing complex. These precipitates were also analyzed by nondenaturing gel electrophoresis to distinguish free U6 snRNA from a U4/U6 heterodimer that is derived from U4/U6.U5 tri-snRNP following phenol extraction (nota bene, Prp8 is not a component of U4/U6 di-snRNPs or free U6 snRNPs). As shown in Fig. 3D, the Prp8 precipitate from Ntr1-depleted extract contained less U4/U6 heterodimer and more free U6 snRNA (presumably derived from the U2/U5/U6 complex) than the Prp8 precipitate from nondepleted extract. This supports the conclusion from the glycerol gradients that Ntr1 depletion results in a defect in recycling of snRNP and indicates that Prp8 accumulates in a U2/U5/U6 snRNP complex.

Ntr1, Ntr2, and Prp43 cofractionate in at least two distinct complexes. Ntr2, Prp8, and the excised intron release factor Prp43 are among many proteins that have been found in affinity-selected complexes along with Ntr1 (13, 16). To investigate this further, extract from KL4G2T cells containing HA-Ntr1 and Ntr2-TAP was fractionated in a 10 to 30% glycerol gradient. Alternate gradient fractions were immunoprecipitated with IgG-agarose, and the precipitated proteins were analyzed by Western blotting with anti-HA and anti-Prp8 antibodies. (C) KL4G cell extract containing HA-Ntr1 was fractionated in a 10 to 30% glycerol gradient. Alternate gradient fractions were immunoprecipitated with anti-HA agarose, and the precipitated proteins were analyzed by Western blotting with affinity-purified anti-Prp43 antibodies and with anti-Prp8 antibodies. (C) Extracts (without added ATP or pre-mRNA) from galactose-grown (lanes 2 and 5) or glucose-grown (lanes 3 and 6) KL4G2T cells or from BMA-18a (untagged control) cells (lanes 1 and 4) were incubated with IgG-agarose to precipitate Ntr2-TAP, and the precipitated proteins were analyzed by Western blotting with anti-HA and anti-Prp8 antibodies. Note that the Ntr2-TAP protein in whole extract is very poorly recognized by IgG on the blot. IP, immunoprecipitation.
Therefore, as Ntr2 pulls down Ntrl and PrpS and NtrI pulls down Prp8 and Prp43 in the same high-density fractions, it is most likely that these four proteins are present in the same high-molecular-weight complex. In addition, it is notable that the bulk of Ntrl-associated Ntr2 and Prp43 occurs in high-density fractions (Fig. 4A and B, fractions 13 to 21). This represents an Ntrl/Ntr2/Prp43 complex that is not snRNAP-associated. Also, Ntr2-TAP coprecipitated both HA-Ntrl and Prp8 in extract from galactose-grown (nondepleted) KL4G (P_OAL-HA-Ntrl and Ntr2-TAP) cells and coprecipitated in extract from glucose-grown KL4G2T cells, despite the depletion of Ntrl (Fig. 4C, lanes 5 and 6). Thus, although level of Prp8 that coprecipitated with Ntr2 was lower in Ntrl-depleted extract, the association of Ntr2 and Prp8, which is direct or indirect, is not absolutely Ntrl dependent, as is the case for its association with excised intron (Fig. 1B).

Ntrl is required for the association of Prp43 with the excised intron complex. The interaction of Prp43 with the excised intron is only weakly detectable in an in vitro splicing reaction mixture, presumably because it is highly transient (31 and unpublished data). Martin et al. (26) have produced an A123 protein that confers a dominant negative phenotype such that the Prp43_{T123A} protein interferes in trans with the in vitro splicing function of the wild-type Prp43 protein, blocks intron release of the excised intron from the spliceosome (26), blocks the accumulation of postsplicing intron-containing complexes, and the presence of the Prp43_{T123A} protein provides a sensitive assay for the interaction of the Prp43_{T123A} protein with the postsplicing complex. We therefore tested whether the purified, recombinant Prp43_{T123A} protein is able to associate with the postsplicing complex produced in reaction mixtures with Ntrl-depleted and nondepleted extracts. As shown in Fig. 5, the Prp43 protein coprecipitated excised intron RNA only from the reaction mixture with nondepleted extract (Fig. 5, lanes 4 and 6). As a control, HA-tagged Ntrl was affinity selected from nontagged KL4G cell extract that contained a very high concentration of HA-Ntrl. The affinity-selected material was incubated with anti-HA-agarose beads for 1 h at 4°C to elute the HA-Ntrl from the beads. Recombinant His-Tagged Prp43_{T123A} protein was produced in Escherichia coli BL21-CodonPlus(DE3)RII (Stratagene) and purified essentially as described by Martin et al. (26). The various RNA species are indicated diagrammatically on the right as described in the legend to Fig. 1.

**DISCUSSION**

Evidence that the Ntrl and Ntr2 proteins are components of the postsplicing excised intron complex includes the coprecipitation by Ntrl and Ntr2 of the U2, U5, and U6 snRNPs (1A); the coprecipitation of more excised intron than intermediates or products of the pre-mRNA splicing reaction (Fig. 1B); and the association of Ntr2 with Ntrl and Prp8 and the intron release factor Prp43 with high-molecular-weight complex (Fig. 4). Evidence that Ntr2 affects the release of excised intron RNA and dissociates the postsplicing complex includes the reduced splicing activity and accumulation of excised intron in an in vitro splicing reaction mixture with Ntrl-depleted extract (Fig. 2C); the coprecipitation of the accumulated excised intron with Prp43_{T123A} protein provides a sensitive assay for the interaction of the Prp43_{T123A} protein with the postsplicing complex. We therefore tested whether the purified, recombinant Prp43_{T123A} protein is able to associate with the postsplicing complex produced in reaction mixtures with Ntrl-depleted and nondepleted extracts. As shown in Fig. 5, the Prp43 protein coprecipitated excised intron RNA only from the reaction mixture with nondepleted extract (Fig. 5, lanes 4 and 6). As a control, HA-tagged Ntrl was affinity selected from nontagged KL4G cell extract that contained a very high concentration of HA-Ntrl. The affinity-selected material was incubated with anti-HA-agarose beads for 1 h at 4°C to elute the HA-Ntrl from the beads. Recombinant His-Tagged Prp43_{T123A} protein was produced in Escherichia coli BL21-CodonPlus(DE3)RII (Stratagene) and purified essentially as described by Martin et al. (26). The various RNA species are indicated diagrammatically on the right as described in the legend to Fig. 1.

**FIG. 5. Immunoprecipitation (IP) of splicing reaction mixtures**

- Splicing reaction mixtures (50 μl) were assembled with 32P-labeled ACT1 substrate RNA and 0.9 μg of purified recombinant Prp43_{T123A}. For reconstitution of the Ntrl-depleted extract (lanes 3 and 6), the splicing reaction mixture was mixed with 3HA-Ntrl affinity selected from galactose-grown KL4G cell extract (overproducing 3HA-Ntrl). Following incubation for 25 min at room temperature, 90% of each splicing reaction mixture was mixed with 20 μl of affinity-purified anti-Prp43 antibodies immobilized on Sepharose CL4B beads in IPP150. After mixing at 4°C for 1 h, the beads were washed and the RNA was extracted. Precipitated RNA (lanes 4 to 6) and RNA extracted from 10% aliquots of the total reaction mixtures (lanes 1 to 3) were separated on a 7% polyacrylamide-8 M urea gel. For each reconstitution reaction mixture, 10 μl of extract from YPG-arose cells was incubated with anti-HA–agarose beads for 1 h at 4°C and the beads were washed five times with IPP150 buffer and once in 0.6 M potassium phosphate buffer (pH 7) and then incubated with 0.5 μg of HA peptide (Sigma) for 8 h at 4°C to elute the HA-Ntrl from the beads. Recombinant His-Tagged Prp43_{T123A} protein was produced in Escherichia coli BL21-CodonPlus(DE3)RII (Stratagene) and purified essentially as described by Martin et al. (26). The various RNA species are indicated diagrammatically on the right as described in the legend to Fig. 1.
Cef1 (Fig. 2C); the reorganization of snRNPs, including a reduction in U4/U6.U5 tri-snRNPs, and free U5 and U6 snRNPs, and accumulation of U4/U6 di-snRNPs, suggesting that snRNPs regeneration after splicing is defective in Ntr1-depleted splicing reaction mixture (Fig. 3B); the association of the U5 snRNP protein Prp8 with a U2/U5/U6 complex in an Ntr1-depleted splicing reaction mixture (Fig. 3C and D); and the failure of the Prp43 T223A protein to associate with the excised intron in the absence of Ntr1 (Fig. 5). Furthermore, Northern and microarray analyses of pre-mRNA splicing demonstrate that Ntr1 is required for efficient splicing in vivo, as well as in vitro (Fig. 2A and data not shown).

Taken together, these data provide compelling evidence that Ntr1 and Ntr2 are present in the late-stage excised intron-containing spliceosome complex and that Ntr1 is required for the interaction of Prp43 with the excised intron prior to its release and recycling of the splicing factors. While the manuscript was under review, Tsai et al. (38) published data showing that metabolic depletion of Ntr2 resulted in accumulation of pre-mRNA and excised lariat-intron in vivo and that a dimeric complex of Ntr1 and Ntr2 is required for dissociation of spliceosomes and release of lariat-intron in vitro. The data presented here complement and extend that study, showing that Ntr1 is required both in vivo and in vitro for efficient splicing and for Prp43 to function in release of the excised intron and recycling of snRNPs. Tsai et al. (38) found that immunodepletion of Ntr1 quantitatively removed Ntr2 from cell extract and vice versa, indicating that these two proteins form a stable complex. We have shown that, following metabolic depletion of Ntr1, Ntr2 is still present in a complex with Prp8 (Fig. 4C) and with excised intron (Fig. 1B), indicating that Ntr2 can associate with late splicing complexes independently of Ntr1 but is not by itself sufficient to activate the intron release function of Prp43.

Ntr1 was originally named Spp382 after the role of this protein in suppression of the prp38-1 temperature-sensitive growth defect. Prp38 is a tri-snRNP-specific protein that was shown to be dispensable for spliceosome assembly but required for spliceosome maturation (6, 45). The depletion of Prp38 caused a spliceosome maturation defect with accumulation of U4/U6, and the temperature-sensitive prp38-1 mutant strain accumulated unspliced pre-mRNA at the nonpermissive temperature (45). The suppression of prp38-1 by Spp382/Ntr1 may suggest an additional role for this protein at an earlier stage, during spliceosome maturation. Alternatively, ntr1 mutations may alleviate the prp38-1 defect indirectly, for example, by moderating the interaction of Ntr1 with a common interacting factor or altering the rate of spliceosome assembly by affecting the availability of splicing factors.

Near its N terminus, Ntr1 contains a G-patch region (glycine-rich sequence found in nucleic binding proteins [2], amino acids 61 to 106) that is the most highly conserved region of this protein. The mammalian G-patch protein called TFIP11 (tuftelin-interacting protein) is a possible ortholog of Ntr1. Human TFIP11 has been found in affinity-purified spliceosomes (18, 46), and mouse TFIP11 was reported to affect the alternative splicing of an adenovirus E1A reporter transcript in a transfection assay (44).

Interestingly, yeast Spp2, another G-patch protein and spliceosome component, interacts with the ATP-dependent DExH-box splicing factor Prp2 (33). As this interaction involves the G-patch sequence in Spp2 and is required for the recruitment of Prp2 to the spliceosome prior to the first catalytic step of splicing, it was proposed that Spp2 may be an accessory factor that confers spliceosome specificity on Prp2 (33). Thus, Ntr1 appears to be an analogous accessory factor for Prp43, targeting it to the excised intron complex, in addition to which it might conceivably be a cofactor for Prp43 helicase activity. Tsai et al. (38) showed that the G-patch region of Ntr1 interacted with Prp43 in a two-hybrid assay. Thus, Ntr1 might promote the interaction of Prp43 with the excised intron through an RNA binding activity of the G-patch region. The role of Ntr2 in these proposed functions remains to be determined.

Intriguingly, Prp43 was recently shown to function also in ribosome synthesis and to associate both with ribosome precursor complexes and with mature ribosomes (12, 20, 21). It is not known how Prp43 is targeted to these distinct cellular processes or whether this dual function allows Prp43 to coordinate the regulation of ribosome biogenesis with pre-mRNA splicing, a process that is vital for ribosomal protein synthesis in budding yeast as many ribosomal protein genes contain an intron. It will be interesting to determine whether Ntr1 acts as an accessory factor for both of these processes or interacts with only a fraction of Prp43, conferring splicing specificity and possibly regulating this in response to different metabolic requirements of the cell.

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rp8 mutations that cause human retinitis pigmentosa lead to a U5 snRNP maturation defect in yeast

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Prp8 protein (Prp8p) is a highly conserved pre-mRNA splicing factor and a component of spliceosomal U5 small nuclear nucleoproteins (snRNPs). Although it is ubiquitously expressed, mutations in the C terminus of human Prp8p cause the U5-specific disease retinitis pigmentosa (RP). The biogenesis of U5 snRNPs is poorly characterized. We present evidence for a cytoplasmic precursor U5 snRNP in yeast that lacks the mature U5 snRNP component Brr2p and depends on a nuclear localization signal in Prp8p for its efficient nuclear import. The association of Brr2p with the U5 snRNP occurs within the nucleus. RP mutations in Prp8p in yeast result in nuclear accumulation of the precursor U5 snRNP, apparently as a consequence of disrupting the interaction of Prp8p with Brr2p. We therefore propose a novel assembly pathway for U5 snRNP complexes that is disrupted by mutations that cause human RP.

Nuclear pre-mRNA splicing is an essential housekeeping process in all eukaryotic cells. It is catalyzed by a large ribonucleoprotein complex called the spliceosome, which contains the snRNPs U1, U2, U4, U5 and U6, as well as many non-snRNP proteins. Each snRNP consists of an snRNA, a set of specific proteins, and seven common proteins (or, in the case of U6 snRNP, seven Lsm proteins).

Unexpectedly, mutations in four human snRNP-associated proteins—PRPF8 (ref. 3), PRPF31 (ref. 4), PRPF3 (ref. 5) and P1-1 (also called RP9) (refs. 6, 7)—were found in people with a dominantly inherited form of retinal degeneration: RP. Here, we investigate the role of Prp8p (the yeast ortholog of PRPF8) in U5 snRNP biogenesis in Saccharomyces cerevisiae, and the effect of RP mutations on this process.

Biogenesis of the U snRNPs has been studied extensively in invertebrates. The U1, U2, U4 and U5 snRNAs are produced as precursors in the nucleus by RNA polymerase II and then exported to the cytoplasm, which is facilitated by nuclear cap-binding proteins and export factors CRM1 and PHAX (ref. 8). In the cytoplasm, the editing of the Sm proteins, which is facilitated by the SMN complex, and hypermethylation of the m7G cap to form a 1-methylguanosine (m1G) cap1 generates a bipartite nuclear localization signal (NLS). Import of this core snRNP to the nucleus is highly conserved in both sequence and size (reviewed in ref. 26). It is not known at what stage the U5-specific proteins associate with the core snRNP. The biogenesis of U snRNPs in yeast is less well characterized. No orthologs of PHAX or Snurportin-1 have been identified in S. cerevisiae11,16, which indicates that the nuclear export and re-import pathways may not exist in yeast.

Prp8p is a large (274 kDa in humans) U5 snRNP-specific protein that is highly conserved in both sequence and size (reviewed in ref. 17). It is essential for pre-mRNA splicing and is produced in all mammalian tissues19. The human PRPF8 gene consists of 42 exons that encode a 2,335-residue protein. Mutations in PRPF8 cause a severe form of dominant RP (ref. 3). At least 16 different mutations have been identified so far, including missense changes, premature stops and deletions20-24. These mutations are clustered at the C terminus of the protein in a conserved region within the last exon.

In budding yeast, Prp8p associates with U5 snRNA in two complexes. The simpler Aar2–U5 snRNP (ref. 25) consists of Prp8p, Snu114p, Aar2p, Sm proteins and U5 snRNA. The larger U5 snRNP (ref. 26) lacks Aar2p but instead contains Brr2p, Prp28p, Snu40p and Diblp. It has been suggested that the Aar2–U5 snRNP might represent an intermediate particle in U5 snRNP biogenesis26. Here, we identify a functional NLS in Prp8p and show that in its absence the Aar2–U5 snRNP accumulates in the cytoplasm. In contrast, the nuclear accumulation of Brr2p, a specific component of the larger U5 snRNP, is unaffected. A similar analysis in yeast carrying prp8 mutations that cause RP in humans also shows an increase in the Aar2–U5 snRNP; however, RP Prp8p proteins accumulate in the

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nucleus. We therefore propose a model for U5 snRNP biogenesis and a molecular basis for a splicing defect caused by mutations that lead to RP in humans.

RESULTS
Evidence for a cytoplasmic U5 snRNP
A putative NLS was identified at amino acids 96–117 of Prp8p of S. cerevisiae within a region rich in basic amino acids that is conserved in most eukaryotes. Codons 96–117 of Prp8 are deleted from plasmid pIJ204 (ref. 27) to produce pKLANLS (prp8ΔNLS-3HA), and these plasmids were introduced into yeast strains KL1 and KL3 (Supplementary Table 1 online), in which the chromosomal P_GAL1-PRP8 is strongly repressed in glucose medium. pKLANLS supported only slow growth of these cells on glucose (4.5 h doubling time; data not shown). Immunofluorescence staining of cells showed that full-length Prp8p localizes to the nucleus, whereas ΔNLS-Prp8p is present in both nucleus and cytoplasm (Fig. 1a). When Prp8p96–117 was fused to the N terminus of green fluorescent protein (GFP), this confirmed the GFP-NLS fusion protein to accumulate in nuclei (Fig. 1b), which confirms that this region of yeast Prp8p contains a functional NLS. As there was sufficient nuclear ANLS-Prp8p protein to support cell viability, albeit with very slow growth, there may be another NLS that allows inefficient nuclear uptake of this very large protein. Candidates include another cluster of basic residues in the N-terminal portion of Prp8p (ref. 17) or, by analogy with metazoan systems, the mG cap of U5 snRNA and/or the Sm proteins SmB and SmD1, which also have NLSes.

Next, pIJ204 or pKLANLS was introduced into yeast strains KL1-Aar2, KL1-Brr2p and KL1-Snu114p, in which the indicated protein is tandem affinity purification (TAP)-tagged, and the tagged proteins were observed microscopically. In strains with full-length Prp8p, the localization of Snu114p and Brr2p was nuclear, whereas Aar2p was present in both nucleus and cytoplasm (Fig. 1c–e). In strains with ANLS-Prp8p, Snu114p was delocalized, with a substantial amount present in the cytoplasm (Fig. 1c), whereas Brr2p was consistently nuclear (Fig. 1d). The cytoplasmic staining of Aar2p was also slightly increased with ΔNLS-Prp8p (Fig. 1f), but this effect was difficult to assess. However, when Prp8p was overexpressed, Aar2p became concentrated in the nucleus, whereas upon Prp8p depletion Aar2p was observed throughout the cells (Fig. 1f). This suggests that the nuclear accumulations of Prp8p and Aar2p are linked; Prp8p might mediate the nuclear accumulation of Aar2p, or vice versa.

Prp8p is a component of U4/U6-U5 tri-snRNPs and of free U5 snRNPs. Importantly, ΔNLS-Prp8p coprecipitated the U5 snRNAs almost as efficiently as did full-length Prp8p, but it coprecipitated less U4 and U6 snRNAs (50–60% relative to full-length Prp8p; Supplementary Fig. 1a online), which suggests a defect in the incorporation of ANLS-Prp8p into tri-snRNPs. Such a defect may occur as a consequence of defective U5 snRNPs or fewer U5 snRNPs in the nucleus. Extract prepared from glucose-grown KLI cells that depend on iNLS-Prp8p for viability also showed a lower splicing activity (Supplementary Fig. 1b), which may be caused by the reduced level of tri-snRNPs.

As the U5 snRNAs seemed to be associated as efficiently with ΔNLS-Prp8p as with full-length Prp8p, despite ΔNLS-Prp8p being distributed throughout the cell, we investigated the localization of U5 snRNA. RNA fluorescent in situ hybridization (FISH) showed that U5 snRNA was predominantly nuclear in the presence of full-length Prp8p but gave a substantial cytoplasmic signal in the ΔNLS-Prp8p strain (Fig. 1g). It is therefore likely that ΔNLS-Prp8p is associated with U5 snRNA in the cytoplasm and in the nucleus. In control strains, U5 snRNA was nuclear in both strains (Fig. 1h), as was U6 snRNA (data not shown).

Distinct Aar2- and Brr2p-U5 snRNPs
In biochemical studies, human Brr2p was found stably associated with human Prp8p in the absence of RNA23. However, a U5 RNP has been identified25 that contains Prp8p and Aar2p but not Brr2p. Indeed, we found that TAP-tagged Prp8p pulls down Aar2p (Fig. 2a), but that TAP-tagged Brr2p does not coprecipitate Aar2p from containing either wild-type (WT) Prp8p or ΔNLS-Prp8p (Fig. 2b). We analyzed the distribution of Aar2p, Brr2p and Snu114p-associated complexes using a combination of glycerol gradient centrifugation and anti-HA (hemagglutinin) immunoprecipitation extracts from KLI-Brr2p cells expressing either full-length Prp8p or ΔNLS-Prp8p-3HA. Gradient fractions were incubated with HA agarose, and the precipitates were analyzed by western blotting. Aar2p was present in 13Myc, Snu114p and Aar2p. The results showed that ΔNLS-Prp8p coprecipitated more Aar2p and less Brr2p than Prp8p (Fig. 2). With WT Prp8p, Snu114p was more concentrated in the bottom fractions (Fig. 2b), which contained the Prp8-associated Brr2p but little or no Aar2p. In contrast, with ΔNLS-Prp8p, Snu114p was more evenly distributed between the fractions containing Aar2p (Fig. 2c). Thus, the ΔNLS-Prp8p extract contained Prp8-Snu114p-Aar2p complex and less Prp8-Snu114p than the WT.
Figure 2. The association of U5 snRNA with the Aar2p complex was investigated in cell extracts from KL1-Aar2 containing either Prp8p or ANLS-Prp8p. An increased amount of U5 snRNA coprecipitated with Aar2p from extract containing ANLS-Prp8p compared with full-length Prp8p (Fig. 2a). This supports the conclusion from the gradient fractionation experiment that the level of Aar2p–U5 snRNP complex increased when nuclear accumulation of Prp8p was hindered. Thus, the existence of two distinct Prp8p complexes is supported by our finding that Prp8p was associated independently with Aar2p or with Aar2p in different glycerol gradient fractions, and that Aar2p did not pull down Aar2p. Furthermore, the glycerol gradient results showing that the prp8ΔNLS mutation increased the amount of Aar2p–Prp8p complex and decreased the amount of Br2p–Prp8p complex suggest that these complexes may be in equilibrium. The presence of U5 snRNA in a cytoplasmic Aar2p–Prp8p complex is suggested by the delocalization of U5 snRNA and the association of more Aar2p with U5 snRNA and with Prp8p in prp8ΔNLS cells. Also, although ANLS-Prp8p is substantially delocalized to the cytoplasm, it is nevertheless associated with a similar amount of U5 snRNA compared to full-length Prp8p. We therefore propose that there is a cytoplasmic Aar2p–Prp8p–Snu14–U5 snRNP complex that, once imported to the nucleus, is in equilibrium with the Br2p–Prp8p–Snu14–U5 mature snRNP complex.

Figure 3. Analysis of Br2p interactions. (a) Splicing extract from glucose-starved KL1-Br2p cells carrying pLJ204-noHA (Br2p-TAP, untagged Prp8p) was combined with glycerol gradient fractionated and precipitated with IgG-agarose, and the precipitates were analyzed by western blotting, probing with anti-Snu14p antibodies. (b) RB-G64-BT cells (Prp8p-TAP) and RB-MetB2-BT cells (Prp8p-TAP and PMET-BR2) were grown under inducing conditions (no methionine), and then methionine was added to 10 mM to repress BR2 expression. Cell extracts were prepared at 0, 6.5 and 10 h after addition of methionine (vertical arrows). (c) Extracts prepared as in b were incubated with IgG-sepharose beads, and the pellets were analyzed by western blot analysis, probing with anti-Br2p, anti-Snu114p and anti-Aar2p antibodies.
Addition of methionine to the growth medium causes cell growth to slow substantially after about 10 h (Fig. 3b) as a consequence of Brr2p depletion. Prp8p-TAP precipitation followed by western blot showed that at 6.5 and 10 h following addition of methionine, as Br2p became depleted, less Br2p and more Aar2p was coprecipitated with Prp8p-TAP, whereas the amount of Snu114p associated with Prp8p-TAP was similar (Fig. 3c). This strongly supports an equilibrium between the Aar2p-Prp8p and Br2p-Prp8p complexes.

**U5 snRNP maturation and RP**

As all of the RP-causing mutations affect amino acids in the extreme C terminus of human Prp8p, a region that is highly conserved from yeast to human, we investigated the effects of some of these mutations in the budding yeast Prp8p. Seven missense mutations were tested, each changing an amino acid that is identical in human and budding yeast Prp8p. We previously demonstrated a two-hybrid interaction between Brr2p and the C terminus of Prp8p (ref. 30). This interaction was disrupted by the yeast Prp8p C-terminal peptide with Brr2p. Yeast Prp8p 2 human Prp8p. We therefore used an in vitro binding assay to investigate the effects of seven missense RP mutations 30 . We therefore used an in vitro binding assay to investigate the effects of seven missense RP mutations 30 . We therefore used an in vitro binding assay to investigate the effects of seven missense RP mutations 30 . We therefore used an in vitro binding assay to investigate the effects of seven missense RP mutations 30 . We therefore used an in vitro binding assay to investigate the effects of seven missense RP mutations 30 . We therefore used an in vitro binding assay to investigate the effects of seven missense RP mutations 30 . We therefore used an in vitro binding assay to investigate the effects of seven missense RP mutations 30 . We therefore used an in vitro binding assay to investigate the effects of seven missense RP mutations 30 . We therefore used an in vitro binding assay to investigate the effects of seven missense RP mutations 30 . We therefore used an in vitro binding assay to investigate the effects of seven missense RP mutations 30 . We therefore used an in vitro binding assay to investigate the effects of seven missense RP mutations 30 . We therefore used an in vitro binding assay to investigate the effects of seven missense RP mutations 30 . We therefore used an in vitro binding assay to investigate the effects of seven missense RP mutations 30 . We therefore used an in vitro binding assay to investigate the effects of seven missense RP mutations.
When extracts from prp8-rp5 cells were tested for the ability to splice in vitro, the rate of ACT1 pre-mRNA splicing was slower than in WT extracts (Fig. 4e). In addition, there was a severe splicing defect when reactions were incubated at both lower and higher temperatures (Supplementary Fig. 3 online). Thus the in vitro splicing activity showed the same heat and cold sensitivity as growth of the prp8-rp5 mutant cells. RT-PCR analysis showed a defect in the splicing of ACT1 transcripts in vivo in pr5 and rp6 cells grown at 30 °C that increased substantially at 37 °C (Supplementary Fig. 4 online). In addition, microarray analysis showed a genome-wide splicing defect for rp5 mutant cells incubated at 14 °C or 37 °C and for rp6 mutant cells incubated at 37 °C, which confirms a general splicing defect in vivo (Supplementary Fig. 5 online).

To investigate the effect of the rp5 mutation on the association of Prp8p and Brr2p with snRNPs, extracts from WT and rp5 mutant cells were incubated with anti-Prp8p or anti-Brr2p antibodies, and the precipitated snRNAs were detected by northern analysis. Mutant and WT Prp8ps were associated with similar levels of U5 snRNAs, but mutant Prp8p brought down less U4 and U6 snRNAs, which indicates a defect in the incorporation of mutant Prp8p into U4/U6-U5 snRNPs (Fig. 4d). In contrast, Brr2p was associated with less U5, U4, and U6 snRNAs in the mutant compared with the WT, but the difference was more pronounced for the U5 snRNAs (Fig. 4d). This result is supported by the amounts of snRNAs that were unbound (Fig. 4d) and suggests a defect in the association of Br2p with U5 snRNA in the mutant strain, with most or all of the mutant U5 snRNAs that do contain Brr2p being incorporated into tri-snRNPs.

These results strongly suggest that although the rp5 mutation does not prevent the association of mutant Prp8p with U5 snRNA, it causes a defect in the formation of Br2p-containing U5 snRNPs, which results in reduced amounts of U4/U6-U5 tri-snRNP complexes, which would explain the reduced splicing efficiency.

Extracts from the prp8-rp mutation strains were fractionated by glycerol gradients followed by immunoprecipitation of Prp8p and western blot analysis. Compared with WT Prp8p (Fig. 4e), the prp8-rp mutant proteins were associated with less Brr2p (Fig. 4f–h) and more Aar2p (Fig. 4f–h). Also, in the mutant extracts, the distribution of Prp8p-associated Smu114p was different, with less present in the high-density fractions compared with WT. The effect of the prp8-rp mutation on Br2p associations was then investigated in a similar way. Notably, extract from the mutant strain showed more Br2p in the lighter gradient fractions and less Smu114p coprecipitating with Br2p from the denser fractions (Supplementary Fig. 6 online). As expected, no Aar2p was detected in any of the Br2p precipitates. This supports the conclusion that the prp8-rp5 mutation causes reduced incorporation of Br2p into U5 snRNPs and larger snRNPs.

The effects of the prp8-rp mutations on snRNP formation and splicing resemble those of the prp8ΔNL5 mutation. We therefore used immunofluorescence microscopy to investigate the localization of the least Prp8p-rp mutant proteins. However, unlike the ΔNL5-Prp8p, the Prp8p-rp mutant proteins showed no sign of delocalization from the nucleus (data not shown). Thus, unlike the situation with ΔNL5-Prp8p, the Prp8p-Aar2p complex that accumulates in the prp8-rp mutants is nuclear and therefore has the possibility to interact with Br2p but does so with reduced efficiency as a consequence of the poor association of Br2p with Prp8p-rp mutant proteins.

DISCUSSION

Our data confirm the existence of two distinct U5 snRNP complexes and suggest that U5 snRNP biogenesis has a cytoplasmic phase in yeast similar to that found in metazoan cells. Although the association of Prp8p with cytoplasmic U5 snRNP precursor particles has not been noted in metazoan cells, considering the high conservation of the U5 snRNP components (especially of Prp8p), it seems likely that the pathway of U5 snRNP biogenesis is also conserved. Indeed, a role for Prp8p in the nuclear uptake of U5 snRNP precursor particles could explain why this process is mG cap-independent in Xenopus sp. oocytes.22 Within the nucleus, the conversion of U5 precursor to mature U5 snRNP involves substitution of Aar2p at the C terminus of Prp8p by Brr2p, and presumably the acquisition of the other three U5-specific proteins (Prp28p, Smu40p and Dib1p). The rp mutations residing in the C terminus of Prp8p interfere with U5 snRNP maturation in a different manner than the N-terminal ΔNL5 mutation, causing a defect in the interaction of Prp8p with Brr2p and thereby reducing functional U5 snRNP and U4/U6-U5 tri-snRNP formation in the nucleus (Fig. 5). Although a precursor-product relationship between the Aar2p–U5 complex and the Brr2p–U5 snRNP has not been formally demonstrated, such a relationship is strongly suggested by the finding that (i) the amount of Aar2p–Prp8p complex is increased, and the amounts of Brr2p–Prp8p complex and U4/U6–U5 tri-snRNP complex are decreased as a consequence of the prp8ΔNL5 or the prp8-rp mutation, (ii) the amount of a new form of Brr2p that is not Prp8p-associated is increased by the prp8-rp mutation and (iii) the metabolic deletion of Br2p also causes an accumulation of Aar2p–Prp8p complex.

We showed previously that Prp8pΔ771-2413 associates with Aar2p and that disruption of Prp8p at residue 2,173 by expressing it as two separate polypeptides disrupts its association with Aar2p. Therefore, Aar2p may interact with the C terminus of Prp8p in close proximity to the region of Brr2p interaction. This suggests a potential model for a competitive interaction of Aar2p and Brr2p with Prp8p. Presumably, Aar2p has an as-yet-unknown but important role in the biogenesis of U5 snRNPs, possibly as a chaperone to control the assembly of the mature particle. Therefore the cytoplasmic location for the formation of the precursor U5 particle may be a critical feature of U5 snRNP biogenesis, ensuring that Aar2p associates with Prp8p before it is exposed to Brr2p in the nucleus. Indeed, there is evidence that the prior binding of Aar2p to a C-terminal fragment of Prp8p inhibits the subsequent association of Prp8p with Brr2p in vitro.
These observations support the hypothesis that the rp defect is a consequence of decreased affinity of Brr2p for a mutated C terminus of Prp8p; this decreased affinity results in a defect in the production of mature U5 snRNPs in the nucleus. This further suggests that the normal equilibrium between the Aar2p-U5 complex and the Brr2p-U5 snRNP complex depends, at least in part, on this Prp8p-Brr2p interaction.

The reduced splicing activity in yeast carrying the rp mutations (Fig. 4c) is likely to be a direct consequence of the reduction in the concentration of mature U5 snRNPs. This is presumably the earliest defect in the splicing pathway that is caused by the rp mutations. However, the incorporation of mutant Prp8p into spliceosomes may also affect spliceosome activation by Brr2p and might explain the observed temperature sensitivity of growth and of splicing caused by some of the rp alleles.

Could this reduced splicing activity explain the clinical consequences of RP in humans? The first symptoms of RP are night blindness and loss of peripheral vision, generally beginning in childhood. RP is therefore a late-onset, progressive, degenerative disease rather than a developmental defect. The retina is a complex, specialized, nondividing tissue with high oxygen consumption and an unusually large number of mitochondria, which implies a high metabolic rate (reviewed in ref. 34). Rod and cone photoreceptors turn over their outer segments daily, which is likely to require a high level of expression of both retina-specific and housekeeping genes during a particular period early each day. Therefore, a reduced level of splicing activity may have a cumulative effect that has much more serious consequences in these cells than in other tissues.

However, mutations causing human inherited disease have been identified in other components of the splicing machinery32,33, each of which may also cause mild splicing defects, and it is unclear what makes the effect of the rp mutations retina-specific. The fact that these cause a dominant genetic defect in humans may offer a clue. The rp5 and rp6 mutations did not cause a dominant growth defect in heterozygous diploid yeast (data not shown). Therefore, the dominant aspect of the disease may be specific to retinal cells. How could our model explain dominance? The accumulation of the Aar2p-U5 snRNP in the nucleus might cause a dominant defect if it became inappropriately incorporated into tri-snRNPs or spliceosomes. Alternatively, accumulation of defective Aar2p-U5 snRNP might sequester a specific factor required for its conversion to the mature U5 snRNP. Also, this factor may be limiting in retinal cells, or the splicing of certain retinal-specific transcripts may involve a retinal-specific factor (in particular a U5 snRNP or U4/U6-U5 tri-snRNP component) that is more sensitive to this defect. It will be important to further characterize the process of U5 snRNP maturation and the specific function of the Aar2p protein.

In the meantime, the results presented here explain how RP mutations can cause a splicing defect and suggest testable hypotheses for the molecular basis of splicing factor RP in human cells.

METHODS

Yeast strains and plasmids. Yeast strains and plasmids used in this work are listed in Supplementary Table 1. Oligonucleotides are in Supplementary Table 2 online. pKLANS was constructed by deleting codons 96-117 via site-directed mutagenesis of PR8 in pJU204 (ref. 27) using oligos F-NLS-P8-352 and R-NLS-P8-285. Strain KL1 has a chromosomal pGALI-GST-pp8aa178 allele that encodes Prp8p with the nonessential N-terminal proline-rich region (amino acids 1-78) missing, thereby allowing use of anti-8.6 antibodies32 to detect only plasmid-encoded Prp8p. KL1 and KL3 cannot grow in glucose medium in the absence of plasmid-encoded PR8p, as the chromosomal PR8p is strongly repressed. KL1 and KL3 were generated from BMA38a by PCR gene replacement using pFA6a-kamMX6-pGALI-GST or pFA6a-pGALI-GST as template36. Strains KL1-Snuil4, KL1-Brr2p and KL3-Brr2p were derived from KL1, and KL3-Brr2p was derived from KL3 by PCR37. The C terminus of PR8p (encoding amino acids 2,173-2,413) was tagged from pJU204 was cloned next to a hygromycin B marker in pCP28. By site-directed mutagenesis, two series of rp mutant plasmids were produced in pCP28 (pCP28-rp1-pCP28-rp7) and in pJU204 (pKL-rp7). The RP mutations were introduced into the genomic PR8p YCL46 (ref. 38). Yeast strains and plasmids used in this work were backcrossed to wild-type cells at least twice to confirm the identity of the inserted alleles.

Microscopy. GFP fluorescence was detected in BMA38a cells carrying pGFP-N-FUS (ref. 39) or pGFP-N-NLS and grown in SD-Ura-immunofluorescence microscopy, cells grown to 0.3-0.5 optical cell density (OD600 nm) were fixed with 1/10 volume 37% (v/v) formaldehyde for 30 min, washed 3 times with buffer B (0.1 M potassium phosphate, 1.2 M sorbitol) and resuspended in 0.5 ml buffer B with lysis for 30 °C. Cells were collected and washed once with buffer B. Microscopy sections were stained on ice with 0.2% (w/v) DAPI (4',6-diamidino-2-phenylindole) in a humid chamber. After washing twice with buffer B, cells were resuspended in 500 μl of buffer B, and 100 μl was added to a polycarbonate slide and left to adhere by incubating for 30 min at 4 °C. Cells were washed once with ice-cold buffer B and stored in 70% (v/v) ethanol for 1 h at 23 °C in the dark, washed with PBS and stained with Alexa Fluor 594 secondary antibodies (Molecular Probes, 1:1000 dilution) for 1 h at 23 °C in the dark. Samples were mounted with Vectashield containing DAPI (4',6-diamidino-2-phenylindole). The U2 and U6 PISH Fisher previously described62, U2, U5, and U6 PISH probes were 5′-Cy3-oligos as listed in Supplementary Table 2.

Immunoprecipitation, northern analysis and glycerol gradient fractionation. Yeast cell extracts were prepared62 and splicing reactions were performed using as substrate 32P-labeled p283 (ACTT) transcript produced by transcription. Immunoprecipitation of snRNPs and spliceosomes performed using rabbit anti-Prp8p polyclonal antibodies (anti-8.6 and anti-8.8) and washing containing 150 mM NaCl. The immunoprecipitates were depicted by SDS/polyacrylamide gel electrophoresis, followed by autoradiography. Strains were hybridized with 32P-labeled oligonucleotides used to detect snRNAs in northern blots

RNA fluorescent in situ hybridization. The method was adapted60 from Singer laboratory protocol (http://www.singerlab.org/protocols/insitu_yeast/). Yeast cells were grown in 36-ml cultures to early log phase (OD600 0.2 and 0.4) and then fixed for 10 min at room temperature (21-23°) directly adding to the medium 8% vol of 20% (v/v) formaldehyde, 5% acetic acid. The fixative was removed by three washes with PBS and ice-cold (0-4 °C) buffer B and centrifugation (5 min at 1,100g, 4 °C). Cells were rehydrated for 5 min in 2x SSC, 50% (v/v) formamide at 37 °C. The U2 and U6 transcripts were washed with 2x SSC, 50% (v/v) formamide, 1 μl RNase A and DAPI (10 ng). Cells were washed twice for 30 min in 2x SSC, 50% (v/v) formamide at 37 °C (U2 probe) or 45 °C (U5 and U6 probes) and resuspended in 500 μl of buffer B. Cells were fixed with 10% for 15 min at room temperature (21-23°) followed by 10% formaldehyde and 1% glutaraldehyde fixation for 1 h at 4 °C. The fixed cells were prepared for I h at 23 °C in the dark, washed with PBS and stained with Alexa Fluor 594 secondary antibodies (Molecular Probes, 1:1000 dilution) for 1 h at 23 °C in the dark. Samples were mounted with Vectashield containing DAPI (4',6-diamidino-2-phenylindole). The U2 and U6 PISF Fisher previously described62, U2, U5, and U6 PISF probes were 5′-Cy3-oligos as listed in Supplementary Table 2.

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d without the rp mutations (data not shown); J.D. Barrass performed the RT

Table of Contents

3. Results

8238 (EURASNET Network of Excellence). J.D. Beggs is the Royal Society

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1. Introduction

2. Materials and methods

3. Results

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