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DISSERTATION

Titel der Dissertation

The role of *Schizosaccharomyces pombe* cyclophilin Rct1

in coupling splicing to RNA polymerase II transcription

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POĎAKOVANIE

Ďakujem všetkým svojim najbližším, čo ma neúnavne, aj únavne podporovali, keď mi bolo najhoršie, zdvíhali hlavu, keď pozerala k zemi, a počúvali, aj keď sa im nechcelo. A hlavne Jurinovi, bez ktorého by som tieto riadky nepísala.

Takže ešte raz, ďakujem mami, tati, Majči, Juri aj Šuli.

.

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to my colleagues for help and support,

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to my family for love.

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ABSTRACT

RNA polymerase II (RNAPII) is a multiprotein complex responsible for transcription of messenger RNAs and some small non-coding RNAs. The largest subunit of RNAPII Rbp1 harbors an extended C-terminal domain (CTD) that is reversibly phosphorylated in transcription cycle-dependent manner. Phosphorylation of the CTD is a dynamic process that regulates transcription and coordinates it with pre-mRNA processing. It has been shown previously that multidomain peptidyl-prolyl isomerase Rct1 from *Schizosaccharomyces pombe* is associated with transcriptionally active chromatin and that it regulates transcriptional activity of RNAPII. Here I investigated a potential role of Rct1 in coupling RNAPII transcription and pre-mRNA splicing.

I demonstrate that Rct1 interacts with the fission yeast SR proteins, Srp1 and Srp2, SR protein-like splicing factor Prp2 ($U2AF^{LG}$) and SR protein-specific kinase Dsk1 *in vitro*. I show that Rct1 employs the RS domain for all of the interactions, but also the PPIase domain for the interactions with Srp2 and Prp2. Similarly, Srp2 and Prp2 employ the RS domain for the interactions, whereas the RS domain of Srp1 is not required. By chromatin immunoprecipitation I show that Rct1 and Srp2 associate with transcritionally active chromatin at *dis2*, *osm1* and *rho1* genes with a distribution pattern similar to RNAPII and that Rct1 RS domain promotes release of RNAPII from the intron-dependent stalling/slow down at *dis2* gene. I also demonstrate that Rct1 RS domain modulates the activity of Rct1 in an heterologous system of the yeast two hybrid assay. As the RS domain of Rct1 interacts with splicing factors Prp2 and Srp2, which also create a complex with RNAPII *in vitro*, I propose a working model where Rct1 negatively regulates RNAPII processivity in an intron-dependent manner and promotes release of RNAPII from the intron-dependent stalling/slow down in Prp2 and/or Srp2-dependent manner. For the first time I also show that endogenous Srp1 and Srp2 proteins are entirely nuclear and prevalently phosphorylated proteins. In accordance with proposed function of Srp1 in sequestration of Srp2, I did neither observe Srp1 to be associated with the transcriptionally active chromatin, nor to create the complex with RNAPII. Finally, in the yeast two hybrid screen I identified a novel Rct1 interacting protein named translin (Tsn1). Although the function of Tsn1 in fission yeasts remain elusive, I show that Tsn1, like Rct1, associates with transcriptionally active chromatin and interacts with splicing factor Prp2 and splicing related kinase Prp4, suggesting that it might be also involved in the regulation of co-transcriptional splicing.

ZUSAMMENFASSUNG

Die RNA Polymerase II (RNAPII) ist ein Multiprotein-Komplex der für die Transkription der *messenger* RNAs (mRNA) und einiger kleiner, nicht kodierender RNAs verantwortlich ist. Die größte Untereinheit der RNAPII, Rbp1, enthält eine verlängerte C-terminale Domäne (CTD), die abhängig vom Transkriptions-Zyklus reversibel phosphoryliert ist. Die Phosphorylierung der CTD ist ein dynamischer Prozess, der die Transkription reguliert und diese durch pre-mRNA Prozessierung koordiniert. Es wurde bereits gezeigt, dass die Multidomänen Peptidyl-Prolyl Isomerase Rct1 aus *Schizosaccharomyces pombe* mit transkriptionell aktivem Chromatin assoziiert ist und die transkriptionelle Aktivität von RNAPII reguliert. In dieser Arbeit habe ich die potentielle Rolle von Rct1 in Verbindung von RNAPII vermittelter Transkription und pre-mRNA *splicing* untersucht.

Ich demonstriere dass Rct1 mit den SR Proteinen Srp1 und Srp2, dem SR Protein-ähnlichen *splicing* Faktor Prp2 ($U2AF^{LG}$) und der SR Protein-spezifischen Kinase Dsk1 sich teilender Hefezellen *in vitro* interagiert. Ich zeige dass Rct1 die RS Domäne für alle diese Interaktionen nutzt, aber ebenso die PPIase Domäne für die Wechselwirkung mit Srp2 und Prp2. Ebenso nutzen Srp2 und Prp2 die RS Domäne für die Interaktionen, wohingegen die RS Domäne von Srp1 nicht benötigt wird. Mit Hilfe von Chromatin-Immunopräzipitation zeige ich dass Rct1 und Srp2 mit transkriptionell aktivem Chromatin an *dis2*, *osm1* und *rho1* Genen assoziiert ist. Das Verteilungsmuster ähnelt dem von RNAPII. Des Weiteren zeige ich dass die RS Domäne von Rct1 die Freisetzung von RNAPII bei der Intron-abhängigen Verzögerung der Transkription am *dis2* Gen fördert. Ich stelle auch dar dass die RS Domäne von Rct1 dessen Aktivität in einem Heterologen System des *yeast two hybrid assay* moduliert. Da die RS Domäne von Rct1 mit den *splicing* Faktoren Prp2 und Srp2 interagiert, welche auch *in vitro* einen Komplex mit RNAPII bilden, erstelle ich ein Modell in welchem Rct1 die RNAPII Prozessivität in Abhängigkeit von Introns negativ reguliert und die Freisetzung von RNAPII an Introns mit verlangsamter Transkription in Abhängigkeit von Prp2 und/oder Srp2 begünstigt. Außerdem zeige ich zum ersten Mal, dass endogene Srp1- und Srp2-Proteine vollständig im Zellkern lokalisiert und häufig phosphoryliert sind. In Übereinstimmung mit der angenommenen Funktion von Srp1 zur Sequestrierung von Srp2 habe ich Srp1 weder in Assoziation mit aktiviertem Chromatin, noch im Komplex mit RNAPII beobachtet. Darüber hinaus habe ich im *yeast two hybrid screen* ein neues mit Rct1 interagierendes Protein, genannt translin (Tsn1), identifiziert. Obwohl die Funktion von Tsn1 in sich teilender Hefe unklar bleibt, zeige ich dass Tsn1, wie Rct1, mit transkriptionell aktivem Chromatin assoziiert ist. Ebenso interagiert Tsn1 mit dem *splicing* Faktor Prp2 und der *splicing*-verwandten Kinase Prp4, was nahe legt dass Tsn1 ebenfalls an der Regulierung von co-transkriptionellem *splicing* beteiligt ist.

CONTENT

ABBREVIATIONS

::	genomic fusion
3-AT	3-amino-1,2,4-triazole
AD	activation domain
Ala	alanine
Asn	asparagine
ATP	adenosine tri-phosphate
BD	DNA-binding domain
BSA	bovine serum albumin
cDNA	coding DNA
ChIP	chromatin immunoprecipitation
CIP	calf intestine phosphatase
CLIP	cross-linking and immuno-precipitation
CPF	cleavage/polyadenylation factor
CsA	cyclosporin A
CTD	C-terminal domain of RNAPII
DMSO	dimethylsulphoxide
dNTPs	deoxynucleotide triphosphates
DRB	5,6-dichloro-1-*b*-D-ribofuranosylbenzimidazole
EDA	spliceosomal exon definition complex A
EDE	spliceosomal exon definition complex E
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ESE	exonic splicing enhancer
ESS	exonic splicing silencer
FKBP	FK506 binding proteins
GE	geneticin
GFP	green fluorescent protein
Gly	glycine
GST	glutathione S-transferase
GTP	guanosine tri-phosphate
H	hygromycin
H3K36me3	histon H3 lysine 36 trimethylation
H3K4me3	histon H3 lysine 4 trimethylation
HA	hemagglutinine
His	histidine
HIV	human immunodeficiency virus
hnRNP	heterogenous nuclear ribonucleoproteins
hs	*Homo sapiens*
Ile	isoleucine
IPTG	isopropyl-β-D-thio-galactoside
mRNA	messenger RNA

NaOAc	sodium acetate
NELF	negative elongation factor
NLS	nuclear localization signal
nmt	no message in thiamine, here the name of repressible promoter
OD_{600}	optical density at λ 600 nm
ORF	open reading frame
PCR	polymerase chain reaction
PEG	polyethylene glycol
Phe	phenylalanine
PPIase	peptidyl prolyl isomerase
pre-mRNA	precursor of messenger RNA
Pro	proline
P-TEFb	positive transcription elongation factor b
PTPA	protein phosphatase 2A phosphatase activator
PVDF	polyvinylidene fluoride
Py	polypyrimidine tract
qRT-PCR	quantitative real-time PCR
RBD	RNA-binding domain
rDNA	ribosomal DNA
RNAPII	RNA polymerase II
RRM	RNA recognition motif
RS domain	domain rich in arginines and serines
RT-PCR	reverse transcription PCR
sc	*Sacharomyces cerevisiae*
SCP	small CTD phosphatase
SDS	sodium dodecyl sulfate
SELEX	systematic evolution of ligands by exponential enrichment
Ser	serine
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
sp	*Schizosaccharomyces pombe*
TCA	trichloro acetic acid
TFIIH	transcription factor IIH
Thr	threonine
Tyr	tyrosine
U2AF	U2 snRNP auxiliary factor
UTR	untranslated region
Val	valine
wt	wild type
XXX	name of gene *XXX* in *S. cerevisiae*
xxx	name of gene *xxx* in *S. pombe*
Xxx	protein, product of gene *XXX* in *S. cerevisiae* or in *xxx S. pombe*
xxx^{+}	wild type allele of gene *xxx* in *S. pombe*

xxx-T	protein Xxx tagged with tag T in *S. pombe*
xxx-Y	gene *xxx* mutated by Y mutation in *S. pombe*
Δxxx	deletion of gene *xxx* in *S. pombe*
YFP	yellow fluorescent protein
ψRRM	pseudo RRM

1. INTRODUCTION

1.1. RNA POLYMERASE II

The RNA polymerase II (RNAPII catalyzes synthesis of mRNA molecules and some small non-coding RNAs using one strand of DNA as a template. The core RNAPII is composed of 12 subunits (Rpb1-Rpb12) that are structurally and functionally conserved from yeast to human (Mitsuzawa and Ishihama, 2004). The largest subunit of RNAPII Rpb1 has globular N-terminal catalytic domain and unique C-terminal domain (CTD) that protrudes out of the catalytic core just below the RNA exit point (Meinhart et al., 2005; Cramer et al., 2004). The CTD of RNAPII is largely unstructured and composed of a number of Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 heptad peptide repeats. The exact number of repeats varies among different organisms (reviewed in Chapman et al., 2008). For instance there are 26 repeats in fission yeasts and 52 repeats in humans, but in humans only 26 repeats harbor the consensus sequence, whereas the C-terminal repeats are highly degenerated. The Ser2, Ser5 and Ser7 of CTD are phosphorylated in transcription cycle-dependent manner and the phosphorylated CTD then serves as a binding platform for many regulatory proteins.

1.1.1. RNAPII transcription cycle

The RNAPII mediated transcription cycle has three different stages: initiation, elongation and termination controlled by different regulatory factors (reviewed in Palancade and

Bensaude, 2003; Phatnani and Greenleaf, 2006; Cho EJ, 2007; Egloff and Murphy, 2008; Margaritis and Holstege, 2008; Buratowski S, 2009). The current working model of RNAPII mediated transcription (see Fig. 1.1., Egloff and Murphy, 2008; Buratowski S, 2009) is as follows: Prior to the transcription, the nonphosphorylated RNAPII associates with DNA and the Mediator (reviewed in Margaritis and Holstege, 2008) in the transcription pre-initiation complex. The Mediator is a multiprotein complex that interacts with DNA regulatory elements and guides the RNAPII to the promoter. Once at the promoter the RNAPII awaits for appropriate signals to start transcription. Upon receiving the initiation signal the Ser5 of CTD becomes phosphorylated, the RNAPII interactions with the Mediator are disrupted and the RNAPII starts initial phase of transcription, so called promoter escape. At this stage RNAPII transcribes only a short sequence and then it pauses. The release from this promoter-proximal paused state requires the phosphorylation of Ser2, the key determinant of productive elongation phase. The major part of productive elongation is characterized by both Ser5 and Ser2 phosphorylations, whereas towards the end of gene the Ser2 phosphorylation becomes the prevalent CTD modification (Kim et al., 2010). The Ser2 phosphorylation is also required for efficient termination and togethr with the Ser5 phosphorylation must be removed prior to another round of transcription.

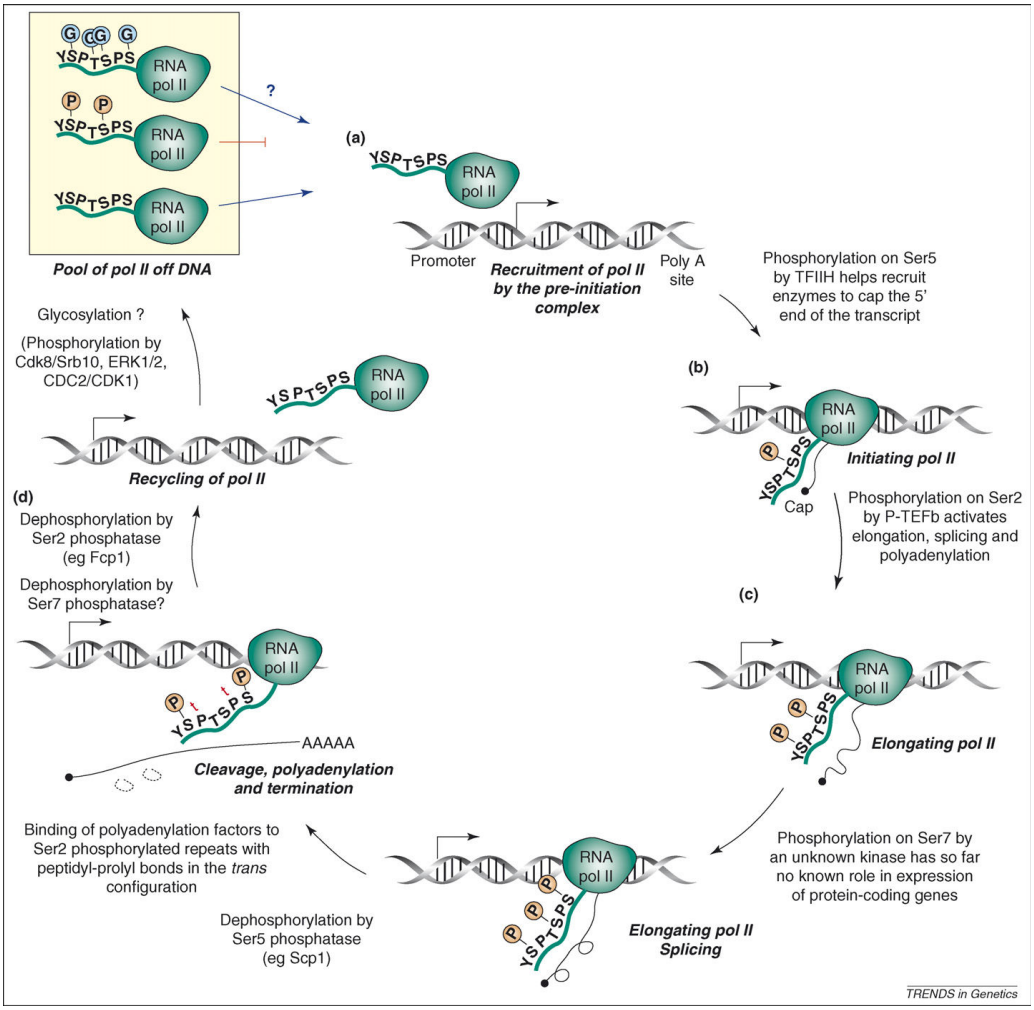

Figure 1.1. Schematic representation of RNAPII transcription cycle according to Egloff and Murphy (2008).

Prior to the initiation of transcription the dephosphorylated RNAPII is recruited to gene's promoter by help of pre-initiation Mediator complex (a). Upon intitiation the TFIIH transcription factor phosphorylates Ser5 of RNAPII CTD and 5' end of nascent pre-mRNA is capped (b). After completion of this first step of transcription Ser2 of CTD is phosphorylated by P-TEFb transcription factor and RNAPII enters the productive elongation phase of transcription (c). Ser7 of CTD also becomes phosphorylated at the beginning of the elongation phase, however, the function of this modification in transcription of protein coding genes is unknown. After completion of the elongation phase the Ser5 and Ser2 are dephosphorylated and RNAPII is released for another round of transcription (d). As indicated in the scheme, different steps of transcription are coupled to different RNA processing activities.

This working model is well consistent with the observation that RNAPII is pre-associated with gene's promoters in the majority of transcription events and waits for appropriate signals to begin the transcription (reviewed in Fujita and Schlegel, 2010). This often leads to cryptic transcription of first bases producing transcripts that are immediately degraded. Controlled switch from the cryptic transcription to the productive elongation enables cell to respond promptly to environmental stimuli and metabolic demands (reviewed in Margaritis and Holstege, 2008). Transcription is usually provided by more than one RNAPII complex on a gene at the same time, further increasing the amount of RNA product generated per time. During the transcription the phosphorylated RNAPII CTD domain is recognized by a number of RNA processing factors that couple the mRNA synthesis with downstream RNA processing pathways, e.g. capping, splicing, polyadenylation, in a well organized sequential order (reviewed in Cramer et al., 2001; Millevoi and Vagner, 2010; Pirngruber et al., 2009a; Cowling VH, 2009; Lenasi and Barboric, 2010; Lunde et al., 2010). The associated factors also modify the chromatin structure along the transcribed gene to facilitate the progression of next RNAPII enzyme (Kundu et al, 2007). It is also plausible that the looping of the template DNA enables the recruitment of terminating RNAPII complex back to the promoter further increasing the overall rate of transcription (reviewed in Brickner JH, 2009; Moore and Proudfood, 2009).

1.1.2. Regulation of RNAPII transcription by CTD domain modifications – the "CTD code"

The main modification of RNAPII CTD is the transcription cycle-dependent Ser2 and Ser5 phosphorylation. It is regulated by several factors, positive and negative transcriptional regulators. While the positive regulators act mainly to enhance the Ser5 and Ser2 phosphorylation, the negative regulators inhibit it. There are several kinases implicated in the phosphorylation of RNAPII CTD. The main kinase responsible for Ser5 phosphorylation and transcription initiation is *hs*CDK7/*sc*Kin28/*sp*Mcs6 cyclin-dependent kinase, the component of general transcription factor TFIIH (Hengartner et al., 1998; Rickert et al., 1999; Watanabe et al., 2000). The CDK7 creates complex with *hs*cyclin H/*sc*Ccl1/*sp*Mcs2 and requires *hs*MAT1/*sc*Tfb3/*sp*Pmh1 co-factor to phosphorylate the RNAPII CTD (Watanabe et al., 2000). The Ser5 can be also phosphorylated by *hs*CDK8/*sc*Srb10/*sp*Srb10 kinase that is associated with the Mediator in the transcription pre-initiation complex (Rickert et al., 1999; Blazek et al., 2005). The mammalian CDK8 can either induce transcription by CTD phosphorylation (Hengartner et al., 1998) or repress the transcription by phosphorylation of cyclin H in TFIIH transcription factor (Akoulitchev et al., 2000; Knuesel et al., 2009). On the contrary, in yeasts the Srb10 kinase, an homologue of CDK9, represses transcription of RNAPII by CTD phosphorylation (Hengartner et al., 1998). Although the mechanisms of CDK8 and Srb10 mediated

initiation seems to be different, it is obvious that CDK8/Srb10 kinase is important for transcription initiation.

The phosphoryaltion of Ser2 is catalyzed by positive elongation factor P-TEFb (Marshall et al., 1996; Lee and Greenleaf, 1997). Up to date there has been only one P-TEFb complex known in metazoans, composed of CDK9 kinase and cyclin T1. By contrast in yeasts two kinases homologous to CDK9 were identified and implicated in Ser2 phosphorylation (Wood and Shilatifard, 2006). The budding yeast kinase Bur1 in complex with cyclin Bur2 was determined to phosphorylate the promoter-proximal Ser2 residues in Kin28-dependent manner and this initial Ser2 phosphorylation was shown to stimulate Ctk1/Ctk2 kinase/cyclin complex to phosphorylate promoter-distal Ser2 residues (Keogh et al., 2003; Qiu et al., 2009). Both Bur1/Bur2 and Ctk1/Ctk2 complexes have homologues in fission yeasts, the Cdk9/Pch1 and Lsk1/Lsc1, respectively (Pei and Shuman, 2003; Karagiannis and Balasubramanian, 2007). Very recently, kinases homologous to Ctk1/Lsk1 were identified in *Drosophila* (dCDK12) and humans (hCDK12 and hCDK13) and were shown to harbour the same promotor-distal Ser2 specificity (Bartkowiak et al., 2010). The main negative regulators of Ser2 phosphorylation are DRB sensitivity-inducing factor (*hs*DSIF/*sc*Spt5/*sp*Spt5) and negative elongation factor (NEL). They are both associated with paused RNAPII and released upon the phosphorylation by CDK9 (reviewed in (Pirngruber et al., 2009a; Fujita and Schlegel, 2010). The CTD of

template-free RNAPII can be also phosphorylated on Ser5 and Ser2 by *hs*CDC2/*sc*Cdc28/*sp*Cdc2 cyclin-dependent kinase and on Ser5 by *hs*ERK-1/2 kinase, resulting in inactive hyperphosphorylated form of RNAPII (Egloff and Murphy, 2008).

Also Ser7 of CTD can be reversibly phosphorylated by *hs*CDK7/*sc*Kin28/*sp*Mcs6 kinase at the promoter-proximal regions (Akhtar et al., 2009; Glover-Cutter et al., 2009) and Bur1 kinase at the promoter-distal regions (Tietjen et al., 2010). The Ser7 phosphorylation was detected along the whole transcriptional units, but it was enriched at the 5' ends of genes and on introns suggesting that it might be involved in the splicing regulation (Kim et al., 2010). Moreover, the Ser7 phosphorylation, as well as the Ser2 phosphorylation, are necessary for recruitment of snRNA gene-specific Integrator complex to the CTD (Egloff et al., 2010) and the Ser7 phosphorylation signal co-localizes with the early termination factor Nrd1 on the snRNA genes (Kim et al., 2010). These results suggest that Ser7 phosphorylation is mainly required for the transcription of non-coding snRNAs, but still might have an yet not defined function in the transcription of mRNA genes.

Beside of serines Tyr1 and Thr4 of CTD can be also phosphorylated. First, it was shown that Thr4 of CTD is phosphorylated by p34 kinase *in vitro* (Zhang and Corden, 1991). Later Baskaran et al. (1993, 1997) showed that the tyrosine kinase encoded by *c-abl* protooncogene phosphorylates Tyr1 on recombinant CTD *in vitro*, while the overexpression of *c-abl* increases Tyr1 phosphorylation and gene transcription *in vivo*.

However, up to date no function has been attributed to the Tyr1 and Thr4 phosphorylations. The mammalian CTD was also found to be glycosilated by the addition of N-acetylglucoseamine to the hydroxyl groups of serine and threonine residues (Kelly et al., 1993). The glycosilation, however, takes place only on the non-phosphorylated CTD, suggesting that it somehow regulates RNAPII recruitment to the promoter. Furthermore, the Ser2-Pro3 or Ser5-Pro6 bonds of CTD can be specifically targeted by peptidyl prolyl *cis/trans* isomerases (PPIases) (Shaw PE, 2007). These enzymes change the conformation of peptidyl-prolyl bonds in a well controlled substrate-specific manner and can fine tune the existing "CTD code" in a very specific way (see part 1.5 for details).

Upon completion of transcription the pSer2 and pSer5 are dephosphorylated by CTD-specific phosphatases and the dephosphorylated RNAPII is then released for another round of transcription (reviewed in Meinhart et al., 2005). The main phosphatase responsible for dephosphorylation of CTD seems to be *hs*Fcp1/*sc*Fcp1/*sp*Fcp1, the component of the transcription factor TFIIH. The human Fcp1 was reported to dephosphorylate both pSer2 and pSer5 with equal efficiency (Lin et al., 2002) or with the preference for pSer5 (Kong et al., 2005), whereas the fission yeast Fcp1 seems to have a preference for pSer2 (Hausmann and Shuman, 2002). The activity of Fcp1 on CTD substrate is further modulated by other CTD-binding proteins (Palancade et al., 2003). For example, it was reported that the PPIase Pin1 (see also part 1.5.) can modulate Fcp1

specificity for pSer2 or pSer5 (Kops et al., 2002). Yeo et al. (2003, 2005) described a family of small CTD phosphatases (SCPs) with catalytic domain structure similar to that of Fcp1, which are involved in the dephosphorylation of pSer5 in humans. Krishnamurthy et al. (2004) identified pSer5 specific phosphatase Ssu72 associated with the cleavage/polyadenylation factor (CPF) complex in budding yeasts. Later an homologue of Ssu72 in humans was described (St-Pierre et al., 2005) and Zheng et al. (2005) discovered ubiquitin-like domain UBLCP1 CTD phosphatase with preference for pSer5. Most recently Mosley et al. (2009) described a novel CTD phosphatase Rtr1 required for pSer5 dephosphorylation during the early elongation phase of transcription in budding yeasts.

1.2. Pre-mRNA SPLICING

1.2.1. Splicing mechanism and regulation of spliceosome assembly

Many pre-mRNA transcripts harbor sequence regions – introns that are not translated into peptide, but are excised of the pre-mRNA transcript in two subsequent transesterification reactions – the splicing reactions. The splicing reactions are catalyzed by the spliceosome, a dynamic ribonucleoprotein (RNP) complex composed of small nuclear RNAs (snRNAs: U1, U2, U3, U4, U5) and many proteins (reviewed in Staley and Guthrie, 1998; Jurica and Moore, 2003; Ritchie et al., 2009; Newman and Nagai, 2010). The snRNAs associate with diverse protein splicing factors and commit the major catalytic activities of the spliceosome. The step-by-step spliceosomal assembly model (Fig.1.2.) is generally accepted (Wahl et al., 2009). In this model the U1 snRNP first recognizes the GU sequence at the 5' splice site of an intron and the U2AF auxiliary factor together with the SF1/BBP protein recognize the branch point, polypyrimidine tract (Py) and the AG dinucleotide at the 3' splice site of the intron creating the prespliceosomal E complex. Then the SF1/BBP is released and U2 snRNP binds to the branch point sequence just upstream of the Py tract resulting in the prespliceosomal A complex. Finally, the U4/U6.U5 tri-snRNP complex is added to the A complex to form the inactive spliceosomal B complex. Prior to the catalysis individual components of the spliceosomal B complex are rearranged and the U1 and U4 snRNPs are released. The remaining snRNPs then form the active spliceosomal complex B* that catalyzes the first transesterification reaction. Again, some re-arrangements occur to form the spliceosomal C complex that catalyses the second transesterification reaction. After the

second transesterification reaction the spliceosome disassembles and the spliced mRNA is released, but still tightly packed with numerous proteins.

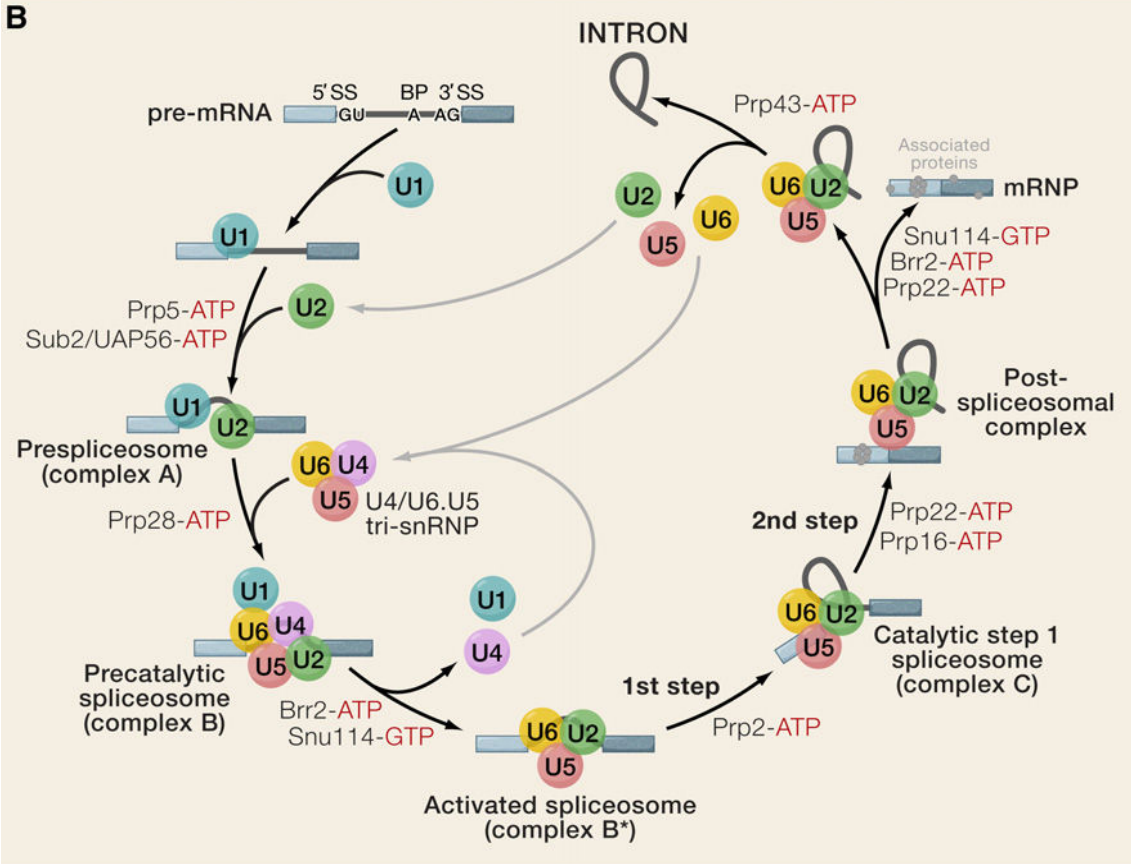

Figure 1.2. Schematic representation of spliceosome assembly according to Wahl et al. (2009).

In the first step of spliceosomal assembly the U1 and U2 snRNPs recognize the 5' and 3' splice sites of target intron in prespliceosomal A complex. Then U4/U6.U5 tri-snRNP complex is added to create precatalytic spliceosomal B complex. After some rearrangements the U1 and U4 snRNPs are released and the spliceosomal B* complex then catalyzes the 1st transesterification reaction. After some additional remodeling the spliceosomal C complex catalyses the 2nd transesterification reaction. Finally, the U2.U4.U6 complex dissociates and spliced mRNA is released in a complex with associated proteins ready for export out of the nucleus. The extensive remodeling of spliceosomal complexes is catalyzed by eight different DExD/H-type RNA dependent ATPases/helicases and one GTPase as indicated in the picture.

The individual spliceosomal complexes can be isolated and the associated RNA splicing intermediates can be visualized. Indeed the majority of spliceosomal subcomplexes were purified and the corresponding protein components were identified. First the core spliceosomal proteins were identified (Neubauer et al., 1998; Rappsilber et al., 2002; Stevens et al.; 2002; Zhou et al., 2002) and later the individual spliceosomal complexes were defined more thoroughly: the pre-spliceosomal A complex (Behzadnia et al., 2007), the spliceosomal B* complex (Makarova et al., 2004; Deckert et al., 2006; Bessonov et al., 2008) and the spliceosomal C complex (Bessonov et al., 2008). In these studies synthetic RNAs with a single intron were used as a matrix for the *in vitro* assembly of the spliceosomal components. This approach likely resulted in the reduced complexity of isolated complexes. Therefore, Chen et al. (2007) purified native mRNA-associated protein complex named supraspliceosome. This complex isolated either form human or mouse cells contained more then 300 proteins. Many of them were well known 5' and 3' end processing factors, RNA export factors and chromatin modifying factors, as well as some proteins with unknown function.

The spliceosomal assembly model described above suggests that the spliceosomal components are rearranged thoroughly during the splicing process. The major driving force for the spliceosomal rearrangements are the DExD/H-type RNA dependent ATPases/helicases that associate with spliceosome at different stages of assembly and facilitate the reorganization of the RNA-RNA and RNA-protein interactions (Staley and Guthrie, 1998; Wahl et al., 2009). Eight helicases (*sc*Sub2/*hs*UAP56, *sc*Prp5, *sc*Prp28/*hs*U5-100K, *sc*Brr2/*hs*U5-200K, *sc*Prp2, *sc*Prp16/*hs*PRP16, *sc*Prp22/*hs*HRH1,

*sc*Prp43/*hs*mDEAH9) are well conserved from yeasts to man and are essential for the function of spliceosome (Staley and Guthrie, 1998; Wahl et al., 2009). Up to date only one GTPase Snu114/U5-116 has been identified within the spliceosomal complex (Fabrizio et al., 1997). The other distinct regulatory proteins associated with the spliceosome are peptidyl prolyl *cis/trans* isomerases (PPIases) of cyclophilin family, i.e. the enzymes that catalyze isomerization of peptidyl bonds proceeding proline (reviewed in Mesa et al., 2008). The change of peptidyl prolyl bond from *cis* to *trans* conformation, or *vice versa*, might change the structure and function of the target protein significantly and the PPIases therefore likely contribute to the major spliceosomal rearrangements (see part 1.5. for details). Nevertheless, the precise model of PPIase-dependent spliceosomal remodeling is still missing.

Many spliceosomal components are posttranslationally modified. In humans at least four different kinases, *hs*SRPK1/*sc*Sky1/*sp*Dsk1, *hs*SRPK2, *hs*PRP4/*sp*Prp4 and *hs*Clk/Sty/*sc*Kns1/*sp*Lkh1 act together to phosphorylate different spliceosomal components (Wahl at al., 2009). The SRPK1, SRPK2 and Clk/Sty are characterized as the RS domain-specific kinases (see part 1.3.2. for details). The SRPK1 and SRPK2 are both able to phosphorylate the RS domain of prototypical SR protein ASF/SF2, in accordance with the preference of both kinases for the RS domain (Kuroyanagi et al., 1998; Wang et al., 1998). However, *in vivo* they are components of two distinct spliceosomal complexes. Whereas the SRPK1 associates preferentially with U1 snRNP complex, the SRPK2 creates complex with the U4/U6.U5 tri-snRNP (Mathew et al., 2008). As a part of tri-snRNP complex the SRPK2 phosphorylates the RS domain of PRP28 helicase *in vivo* and as a

consequence regulates the spliceosomal B complex assembly. The *sp*Prp4 was the first kinase identified to phosphorylate a non-SR protein splicing factor, the *sp*Prp1 (Schwelnus et al., 2001). Later Dellaire et al. (2002) showed that the human PRP4 kinase is a component of the U4/U6.U5 tri-snRNP complex and the experiments of Schneider et al. (2010a) revealed that the phosphorylation of *hs*PRP6, *sp*Prp1 and *hs*PRP31 proteins of this complex by PRP4 is necessary for the spliceosome B complex formation. The mouse and fission yeast Prp4 homologues were reported to phosphorylate also the RS domain of ASF/SF2 SR protein (Gross et al., 1997). Interestingly, the human PRP4 protein has RS rich N-terminal domain typical of SR proteins (see also part 1.3.), but the *S. pombe* homologue miss this region. There are few more spliceosomal phosphoproteins reported up to date, however, the information regarding the mechanism of spliceosomal phosphoregulation is only sparse (Mermoud et al., 1992, 1994; Misteli T, 1999). The other protein modifications recognized among splicing factors are ubiquitination and acetylation, but there likely also exist other, yet not detected, modifications (Wahl et al., 2009).

1.2.2. Constitutive and alternative splicing

In protozoa and lower eukaryotes (e.g. yeasts) the majority of introns are constitutively spliced. On the contrary, in the higher eukaryotes splicing results in alternative splicing pattern of many genes (reviewed in Graveley BR, 2000; Pozzoli and Sironi, 2005; Keren et al., 2010). As a consequence different splice variants with different information regarding the function and faith of the mRNA, i.e. information that leads to the mRNA translation,

deposition or decay, are generated (reviewed in Giorgi and Moore, 2007; Houseley and Tollervey, 2009; Moore and Proudfoot, 2009).

Several types of alternative splicing have been described (reviewed in Hertel KJ, 2008; Long and Caceres, 2009; Keren et al., 2010). The most common type of alternative splicing in fungi, protozoa, lower metazoans and plants is the intron retention, whereas the prevalent type in higher eukaryotes is the exon skipping and alternative 3' and 5' splice site selection. Two models have been proposed to describe the intron retention and exon skipping mechanisms (Berget SM, 1995). For the intron retention an intron definition model have been proposed (Fig. 1.3.A). In this model the splicing of an intron is guided by the intron bridging interactions that occur among different splicing factors. The alternative intron is then spliced out employing the mechanism described in the section 1.2.1. The small intron size and some experimental evidence argument for the intron recognition model to be the prevalent mode of splice site recognition in the fission yeasts (Romfo et al., 2000; Haraguchi et al., 2007). For the exon skipping mechanism an exon definition model have been proposed (Fig 1.3.B.). In this case the exon skipping is defined by the interaction network that assembles across an exon. Here, U2AF and U1 snRNP bound to the exon adjacent 3' and 5' splice sites pair together and interact with the U4/U6.U5 tri-snRNP to create the exon definition complex (Schneider et al., 2010b). As a result the splicing machinery recognize only the 5' splice site upstream and 3' splice site downstream of exon and cuts the exon out together with the adjacent introns. If the alternative exon inclusion is appropiate, the exon definition complex is changed to the intron definition complex, i.e. the U2AF of exon definition complex pairs with the

upstream 5’ splice site and U1 snRNP with the downstream 3’ splice site to excise only the intronic sequences and not the exon (Schneider et al., 2010b).

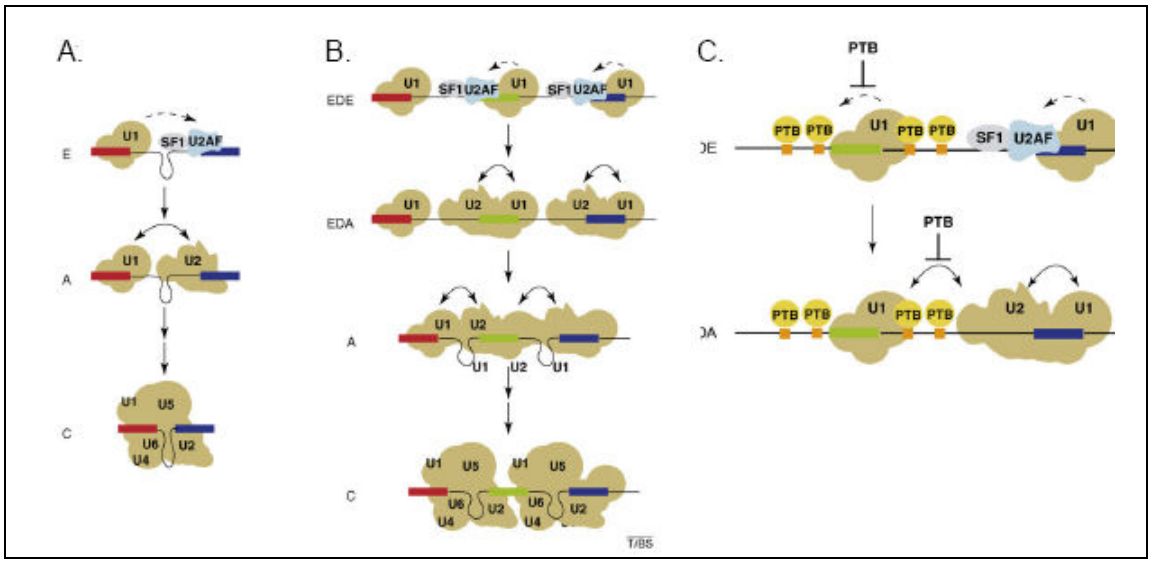

Figure 1.3. Intron and exon definition models according to Schellenberg et al. (2008).

In the intron definition model (a) the 5’ splice site is recognized by U1 snRNP complex and 3’ splice site by SF1/BBP.U2AF[65] complex in the pre-spliceosomal E complex. Later the U2 snRNA complex is bound at the 3’ splice site and the U1 and U2 snRNPs interact with each other in spliceosomal A complex. Finally the intron is spliced out by catalytically active spliceosomal C complex and the two constitutive exons (red and blue) are joined together. In the exon definition model (b,c) the alternative exon (green) can be either spliced out (c) or retained (b) in the mature mRNA. If retained (b), the SF1/BBP.U2AF[65] complex binds at the exon’s 3’ splice site and the U1 snRNP at the exon’s 5’ splice site in the exon definition E complex (EDE). Upon U2 snRNP binding the upstream U2 snRNP interacts with the downstream U1 snRNP complex in the exon definition A complex (EDA). After exon definition complex has been created it is converted to intron definition A complexes over the exon’s adjacent introns and the adjacent introns are spliced out. If the alternative exon is spliced out (c), the exon definition complex is not created and the pairing occurs between the U1 snRNP upstream of alternative exon and U2 snRNP downstream of alternative exon. As a result the alternative exon with the adjacent introns is spliced out. The exon definition complex formation is inhibited by the action of different hnRNP protein like PTB, that interfere with U2AF[65] binding and U1 and U2 snRNP pairing.

In some genes the transition between exon definition and intron definition complex is controlled by PTB factor that inhibits binding of U2AF to the 3' splice site and U1 and U2 snRNP pairing and therefore interfere with the formation of the exon definition complex (Sharma et al., 2005, 2008; for review see Schellenberg et al., 2008, see also Fig. 1.3.C). Although PTB is the best understood factor required for the exon to intron definition transition, it is likely not the only factor required for the regulation of this process.

From the above is clear that the first steps of splicing reaction must be under the tight control of splicing factors to ensure the proper splicing pattern of target genes. It seems plausible that the definitive splice site selection occurs during the spliceosomal A complex formation and it was proposed that the hydrolysis of ATP at this stage of assembly locks the paired 5' and 3' splice sites for the final splicing pattern (Kotlajich et al., 2009). Prior to the final pairing between the U1 and U2 snRNPs a dynamic network of interactions defines the splice sites. There are several strategies employed by different splicing factors and heterogeneous nuclear RNPs (hnRNPs) to select the optimal splice site. In general, these factors recognize the signal RNA sequences adjacent to the putative splice site and communicate the information to the core splicing machinery (reviewed in Pozzoli and Sironi, 2005; Hertel KJ, 2008; Shellenberg et al., 2008; Long and Caceres, 2009; Shepard and Hertel, 2009). The sequences are termed enhancers, if their recognition leads to preferential inclusion of the splice site, or silencers, if they result in skipping of the splice site. The major group of proteins that positively regulate spliceosomal assembly are the SR proteins (see part 1.3. for details), whereas the negative regulation is mainly mediated by the structurally diverse hnRNP proteins

(Pozzoli and Sironi, 2005). Different positive regulators can act in synergy to enhance splicing of a particular intron, but the combined action of the positive and negative regulators is antagonistic (Fig. 1.4.). A final splicing pattern is therefore the result of summed interactions that occur at the particular splice site.

1.3. SR PROTEINS, SR-LIKE PROTEINS AND REGULATION OF SPLICING

The SR proteins are group of splicing factors with the key function in regulation of constitutive and alternative splicing (reviewed in Long and Caceres, 2009; Shepard and Hertel, 2009). Although they were originally identified as splicing factors (Fig. 1.4.), nowadays the functions assigned to them cover many different stages of gene expression, ranging from transcription to translation (reviewed in Moore and Proudfoot, 2009; Zhong et al., 2009).

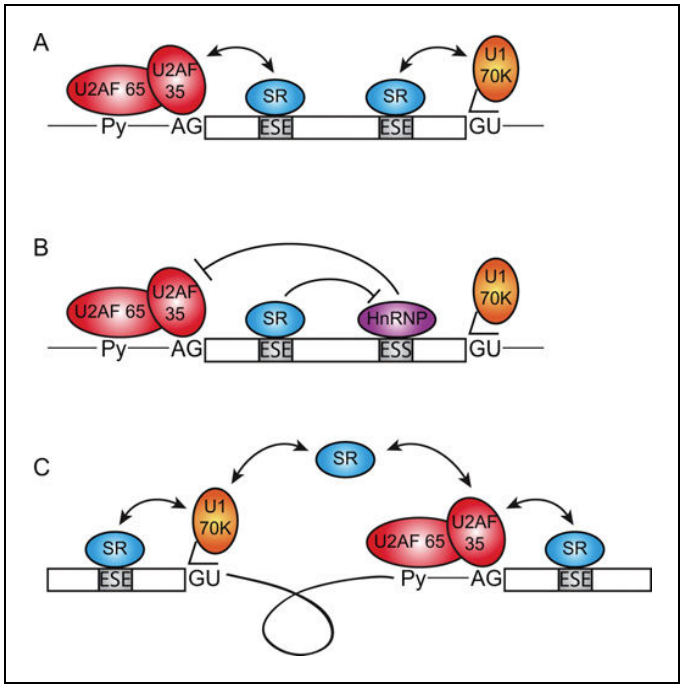

Figure 1.4. Schematic representation of interactions leading to alternative splice site choice according to Long and Caceres (2009).

The interaction of U2AF splicing factor with 3' splice site and U1-70K splicing factor with 5' splice site is regulated by exonic splicing enhancer-bound (ESE-bound) SR proteins that positively regulate the splice site selection (A). Alternatively, the upstream 3' splice site can be attenuated by the interactions with exonic splicing silencer-bound (ESS-bound) hnRNP proteins (B). The splice site selection can be also modulated by free SR proteins that are not, bund to RNA (C).

According to the current definition (Long and Caceres, 2009), all members of this group are characterized by one or two RRM domains and an RS domain and must act as splicing regulators. Proteins with similar structural features, but not involved in the splicing regulation, or proteins with the RS domain, but lacking the RRM domain, are collectively termed SR-like or SR-related proteins and are not considered bona fide SR proteins. Both, SR proteins and SR-like proteins might harbor additional domains with diverse functions (Long and Caceres, 2009).

1.3.1. The RNA recognition motif (RRM domain) and its function

The RNA recognition motif (RRM) domain, also known as RNA binding domain (RBD), is a protein fold consisting of at least four β-strands and two α-helices organized in $\beta_1\alpha_1\beta_2\beta_3\alpha_2\beta_4$ topology (reviewed in Maris et al., 2005; Clery et al., 2008). Based on numerous crystal structures the β-strands create an antiparallel β-sheet spatially arranged in $\beta_4\beta_1\beta_3\beta_2$ order with two α-helices positioned above or below it. There are two highly conserved segments named RNP1 (Lys/Arg-Gly-**Phe/Tyr**-Gly/Ala-**Phe/Tyr**-Val/Ile/Leu-X-Phe/Tyr) and RNP2 (Ile/Val/Leu-**Phe/Tyr**-Ile/Val/Leu-X-Asn-Leu) on β_3 and β_1 strands, respectively, that are essential for RRM-RNA binding. The RNA binding is mediated by aromatic residues of Phe or Tyr side chains (marked in bold) that stack with the RNA bases. The resulting RRM-RNA binding affinity and specificity is usually high, but can be modulated by protein – protein interactions employing the β_2 and β_4 strands, the intervening loops and C-terminal and N-terminal parts of the protein. A single RRM domain can recognize two to eight nucleotides of RNA (Clery et al., 2008); however,

multiple RRM domains often cooperate in RNA binding and recognize longer stretches of RNA (Ding et al., 1999; Handa et al., 1999).

The SR proteins usually employ the RRM domains for recognition of the enhancer or silencer sequences. Many SR protein target sequences were identified employing either SELEX/functional SELEX or CLIP procedures (Liu et al., 1998; 2000; Sanford et al., 2008). Many splicing factors recognize their target sequences individually; however, sometimes two SR proteins can cooperate to enhance the splicing of an alternative exon as was reported for ASF/SF2 and 9G8 on FP/ESE of bovine growth hormone pre-mRNA (Li et al., 2000). Also the phosphorylation status of an SR protein influences the ESE binding. For example Srp40 binds to the enhancer only if it is phosphorylated on the RS domain (Tacke et al., 1997). Furthermore, the RRMs can cooperate with the other domains, for example zinc knuckle domain, in the recognition of the target sequence (Cavaloc et al., 1999). Some SR proteins, including ASF/SF2, have two RRM domains, but only the RRM1 has conserved RNP1 and RNP2 motifs. The RRM2 does not harbor the RNP1 and RNP2, but instead has conserved SWQDLKD motif that contributes to RNA binding (Tacke RMJ, 1995; Tintaru et al., 2007). The RRM1 of ASF/SF2 was implicated in binding to enhancer, whereas the RRM2 seems to be required for alternative 5' splice site selection of adenovirus E1A gene (Dauksaite and Akusjarvi, 2004). Importantly, although the RRM domain is considered to be mainly an RNA binding domain, in some proteins it also participates, or is fully endowed, in the protein-protein interactions as exemplified by RRM3 of $U2AF^{65}$ protein (Kielkopf et al., 2001; Selenko et al., 2003; reviewed in Maris et al., 2005). Therefore, although the RRM domains of SR proteins are mainly responsible

for binding to RNA regulatory elements they can be also involved in the transient protein - protein interactions that occur during the spliceosomal assembly.

1.3.2. The RS domain and its function

The RS domain is usually C-terminal domain of SR proteins characterized by a number of stretches consisting of arginine-serine dipeptides and hence prevalently positively charged. The serines of this domain are targeted by RS domain-specific kinases of SRPK and Clk/Sty families (Stamm S, 2008; Long and Caceres, 2009; Shepard and Hertel, 2009). The phosphorylated RS domain usually harbors nuclear localization signal (NLS) that interacts with the SR protein nuclear import receptor. On the contrary, the dephosphorylation of RS domain is usually required for the efficient nuclear export of shuttling SR proteins (Lin et al., 2005; Tenenbaum and Aguirre-Ghiso, 2005). The phosphorylation is also considered to prevent the spontaneous aggregation of unphosphorylated RS domain proteins (Nikolakaki et al., 2008). It is plausible that the RS domain phosphorylation is a highly ordered process. This is well documented for ASF/SF2 protein (Aubol et al., 2003; Ngo et al., 2005; Velazquez-Dones et al., 2005; Ma et al., 2009). The RS domain of ASF/SF2 can be subdivided into two regions, N-terminal RS1 and C-terminal RS2. The RS1 is phosphorylated by SRPK1 kinase in the cytoplasm and subsequently the protein is transported into the nucleus where it is deposited in the nuclear speckles. The RS2 is phosphorylated by Clk/Sty in the nucleus and as a result the ASF/SF2 is released from the nuclear speckles and associates with the spliceosome. I n the order to become fully active, the RS domain must be transiently dephosphorylated during the splicing process (Mermoud et al., 1992, 1994).

The RS domain is both, protein and RNA interacting domain. The RS-RS domain interactions were detected among several splicing factors (Wu and Maniatis, 1993) and are believed to direct, at least partially, the early steps of spliceosomal assembly (reviewed in Graveley BR, 2000). Three of the key spliceosomal components, U1-70K, $U2AF^{65}$ and $U2AF^{35}$ harbor an RS domain. In the early models of splice site recognition and complex A assembly the enhancer bound SR proteins were suggested to interact through their RS domains with U1-70K, $U2AF^{65}$ and $U2AF^{35}$ splicing factors (Graveley BR, 2000). First Wu and Maniatis (1993) showed that these interactions are RS domain-dependent. Their results were well consistent with later experiments (Zuo and Maniatis, 1996; Graveley et al., 2001) that investigated enhancer-dependent complex E formation at the weak 3' splice site of *doublesex* (*dsx*) gene in *Drosophila*. They showed that the enhancer bound SR proteins (Tra and Tra2) interact with $U2AF^{35}$ that in turn recruits $U2AF^{65}$ to the Py tract. However, some other experimental evidence does not support this model. For example Rudner et al. (1998) showed that the RS domains of either $U2AF^{35}$ or $U2AF^{65}$ subunits in *Drosophila* are dispensable *in vivo* and do not affect the *dsx* pre-mRNA splicing, although the double deletion is synthetically lethal. Similarly, the results of Li and Blencowe (1999) suggest that the $U2AF^{65}$ binding to weak 3' splice site of *dsx* is not enhancer-dependent.

The RS domains of SR proteins were also implicated in the direct interactions with the pre-mRNA/snRNA duplexes (reviewed in Graveley BR, 2004; Hertel and Graveley, 2005) and it was proposed that they promote pairing of the RNA strands and facilitate the spliceosomal rearrangements (Fig. 1.5., Shen and Green, 2006).

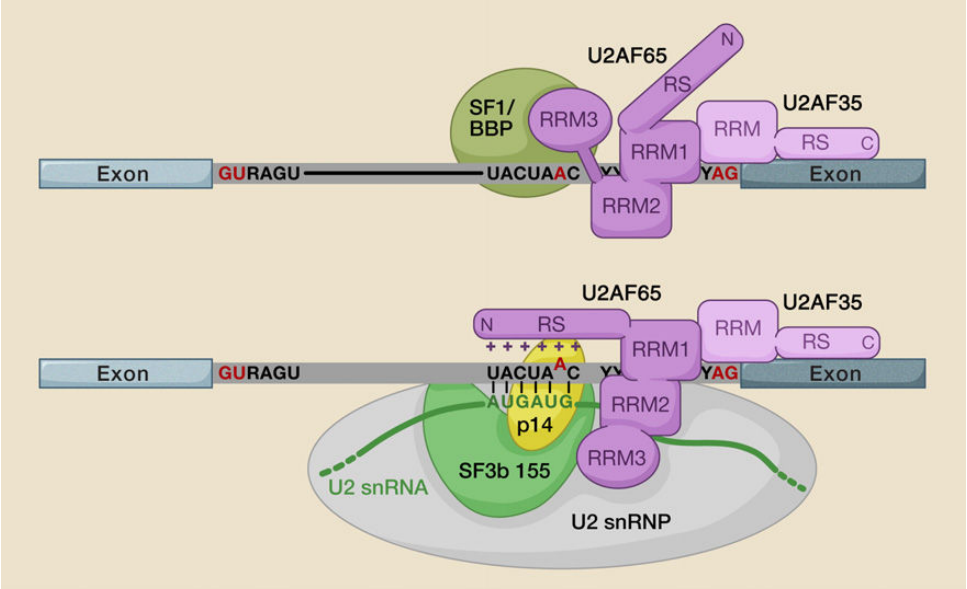

Figure 1.5. Molecular interactions at the branch site and the 3' splice site in the spliceosomal E and A complexes according to Wahl et al. (2009).

In the pre-spliceosomal E complex (top) the SF1/BBP protein (green) occupy the branch site, the U2AF^{35} protein binds to 3' splice site (light pink) and U2AF^{65} (dark pink) binds the Py tract. The U2AF^{65} also interacts with SF1/BBP via the RRM3 domain and U2AF^{35} via the linker region between the RRM1 and RS domain. Upon the binding of U2 snRNP (bottom) the SF1/BBP dissociates and the branch site is recognized by a complementary sequence of U2 snRNA. This RNA-RNA interaction is further stabilized by the RS domain of U2AF^{65} protein. The U2AF^{65} also interacts with the protein components of U2 snRNP via the RRM3 domain.

Valcarcel et al. (1996) showed that the RS domain of U2AF^{65} can bind to the branch point sequence of an intron in the spliceosomal E complex. Later experiments of Shen et al. (2004b) revealed that this interaction is replaced by another RS-RNA interaction in the spliceosomal A complex. At this stage the pre-mRNA/U2 RNA duplex is recognized by the RS domain of an enhancer-bound SR protein, for example ASF/SF2. Another SR protein, this time not bound to the enhancer, associates with the 5' splice site in the spliceosomal C complex together with the U6 snRNA (Shen et al., 2004a). This together with RS-RS

domain protein interactions discussed above increase the complexity of not yet fully understood SR protein-dependent splicing regulation. However, it is plausible that both RS-RS and RS-RNA interactions are important for the spliceosome assembly and splicing regulation. (Shen and Green, 2006; Hertel and Graveley, 2005).

1.3.3. Fission yeast SR proteins and SR-like proteins

Up to date there are two SR proteins, Srp1 and Srp2, characterized in fission yeasts and one predicted SR protein Rsd1. Like in higher eukaryotes the large subunit of U2AF factor Prp2 has structure reminiscent of SR proteins. On the contrary, the small subunit of U2AF factor (*sp*U2AF^{SM}) and the U1-70K protein (*sp*Usp101) do not harbor the RS domains. Furthermore, the RS domains of fission yeast proteins are phosphorylated by at least three different kinases, Dsk1, Lkh1 and Prp4. The structure and activity of Dsk1 and Lkh1 is similar to that of SRPK1 and Clk/Sty1 in the higher eukaryotes (reviewed in Kaufer and Potashkin, 2000; Kuhn and Kaufer, 2003).

Srp1

The nonessential protein Srp1 has one N-terminal RRM domain (designated RBD1 in Gross et al., 1998) with conserved RNP1 and RNP2 motives and one C-terminal RS domain. It was identified as a suppressor of *prp4-73* splicing defective phenotype when overexpressed Gross et al. (1998).

The RS domain of Srp1 can be subdivided into three regions, designated RS1, RS2 and RS3. Tang et al. (1998) showed that the overexpression of truncated Srp1 ΔRS protein,

but not the overexpression of the full length protein in the wild type cells, resulted in the accumulation of unspliced pre-mRNAs and growth defects. Similarly, the mutation of any of the RS regions and subsequent overexpression of the mutated proteins resulted in the same phenotype. These experiments show that every subdomain of Srp1 RS domain is essential for pre-mRNA splicing *in vivo*. Importantly, the deletion of RS domain does not interfere with the correct nuclear localization of Srp1 protein, as might have been expected (Lutzelberger et al., 1999). The RS domain of Srp1 is both, *in vivo* and *in vitro*, phosphorylated by Dsk1 kinase (Tang et al., 2002, 2007), an homologue of human SRPK1 (Tang et al., 1998, 2000) and Lkh1 kinase, a member of Clk/Sty family of kinases (Kim et al., 2001; Tang et al., 2003). Furthermore, the deletion of *dsk1* gene resulted in the partial retention of Srp1 protein in the cytoplasm (Tang et al., 2007) arguing that the Dsk1 mediated phosphorylation is important for Srp1 localization. The genetic interaction between *srp1* and *prp4* (Gross et al., 1998) and the fact that both human and fission yeast Prp4 kinases phosphorylate the RS domain of ASF/SF2 *in vitro* (Alahari et al., 1993; Gross et al., 1997) opens the possibility that the Prp4 kinase is also required for the phosphorylation of Srp1. However, the direct experimental evidence to support this assumption is missing.

From the experiments described above is clear that the overexpression of Srp1 RRM domain in the wild type cells has a dominant negative effect on the cell viability and pre-mRNA splicing. By mutational analysis Gross et al. (1998) showed that the mutation of the highly conserved motif RDAEDA, that is located just upstream of RRM, rescues the deleterious effect of Srp1 RRM domain overexpression. This experiment therefore argues

that the RDAEDA-dependent RRM binding to its substrate has a negative effect on the splicing of the tested RNA.

The only interacting protein found in the yeast two hybrid screen with Srp1 protein as a bait was Srp2 protein (Tang et al., 2002). This interaction was further verified *in vitro* and turned out to be inhibited by Dsk1-dependent Srp1 phosphorylation. Srp1 was also assayed for the interactions with Prp2 *in vitro* ($U2AF^{LG}$, Tang et al., 2002) and $U2AF^{SM}$ in the yeast two hybrid assay (Webb and Wise, 2004), but was not found to interact with any of the two proteins. There is also no experimental evidence for the interaction of Srp1 with any ESE elements (Webb et al., 2005b). Therefore, it was postulated that Srp1 is not directly involved in the splice site selection, but that it likely serves to modulate the activity and localization of Srp2 protein (Tang et al., 2007).

Srp2

The Srp2 is an essential protein with two N-terminal RRM domains, RRM1 and RRM2 (marked as RBD1 and RBD2 in Lutzelberger et al., 1999), and an atypical C-terminal RS domain.

The RRM1 has conserved RNP1 and RNP2 motives, whereas the RRM2 does not. Instead it has a highly conserved SWQDLKD motif also typical for the human SR proteins ASF/SF2, SRp55, SRp75, SRp40 and SRp30 (Dauksaite and Akusjarvi, 2004). The RRM2 domain, in particular the SWQDLKD motif, seems to be the most important for the function of Srp2 protein, because only the RRM2 mutants were not able to rescue the conditional lethal

phenotype of *nmt1-8::srp2*$^{+}$ strain, although the RRM1 and RS mutants did rescue this phenotype (Lutzelberger et al., 1999). Furthermore, the overexpression of proteins with deleted RRM2 domain or with mutated SWQDLKD motif in the wild type strain resulted in the significant growth defects, whereas the overexpression of full length, Srp2 ΔRRM1 or Srp2 ΔRS proteins did not. Interestingly, the overexpression of Srp2 ΔRRM1ΔRRM2 protein did not have any effect on cell growth, suggesting that the RRM2 domain buffers otherwise deleterious effect of RRM1 domain overexpression. This is also the case if RRM1 of Srp2 is replaced with RRM1 of Srp1, suggesting that the RRM2 of Srp2 indeed modulates the activity of adjacent RRM1 domain. Interestingly, the interaction of Srp2 with the non-phosphorylated Srp1 does not depend on the Srp2 phosphorylation status (Tang et al., 2002).

The RS domain has only two short RS dipeptide-rich segments, but if they are mutated the overexpressed proteins are not able to rescue the conditional lethal phenotype of *nmt1-8::srp2*$^{+}$ strain (Lutzelberger et al., 1999). The protein localization studies showed that these segments are important for the nuclear localization of Srp2 protein, explaining the mentioned phenotype. The RS domain of Srp2 is phosphorylated by Dsk1 and Lkh1 kinases (Tang et al., 1998, 2000, 2002, 2007) and the Dsk1-dependent Srp2 phosphorylation seems to be, at least partially, responsible for the nuclear localization of Srp2 protein (Tang et al., 2007).

As already mentioned the Srp2 protein interacts with Srp1 protein *in vivo* and *in vitro* (Tang et al., 2002). Furthermore, Srp2 was found to interact with U2AF^{SM} in the yeast

two hybrid assay (Webb and Wise, 2004) and with purine-rich ESEs in the yeast three hybrid assay (Webb et al., 2005b). The binding of Srp2 to either native *S. pombe* ESEs or the well characterized ESEs of higher eukaryotes well correlated with the enhanced splicing rate of the assayed introns, directly linking the Srp2 protein function with the splicing regulation. In fact one of the Srp2-dependent ESEs in *S. pombe* lies within the exon 6 of *srp2*$^{+}$ gene opening the possibility that Srp2 autoregulates splicing of its own pre-mRNA *in vivo*; however, an evidence for this scenario from *in vivo* experiments is still missing (Webb et al., 2005b).

Prp2

The essential protein Prp2 (U2AF^{LG}) is a component of the core spliceosomal machinery. The *prp2*$^{+}$ gene was originally identified in the genetic screen aimed to identify the factors required for pre-mRNA splicing in *S. pombe* (Potashkin et al., 1989). Later it was determined to be the homologue of the large subunit of U2AF splicing factor (Potashkin et al., 1993). Despite the structural similarity, Romfo et al. (1999) showed that the human U2AF^{65} and Prp2 are not functionally interchangeable. Prp2 has N-terminal RS domain followed by a short linker region, two canonical RRM domains, RRM1 and RRM2, an C-terminal pseudo-RRM domain ψRRM (or RRM3) and all four of them are essential for viability (Webb et al., 2005a).

Similar to other SR proteins the RS domain of Prp2 is phosphorylated by Dsk1 protein kinase (Tang et al., 1998, 2000) and is required for the efficient nuclear localization of the Prp2 protein (Tang et al., 2007). The deletion of RS domain has dominant negative effect

on the cell viability, but does not interfere with spliceosomal E complex formation (Webb et al., 2005b). It is therefore possible that the Prp2 RS domain is required for the later steps of spliceosomal assembly (Webb et al., 2005).

The linker region between the RS domain and RRM1 domain is responsible for the interaction with the $U2AF^{SM}$, the small subunit of U2AF factor (Wentz-Hunter KPJ, 1996; Webb et al., 2005b). The mammalian homologue $U2AF^{35}$ employs the pseudo-RRM for the interactions with the linker region of $U2AF^{65}$ subunit in reciprocal tong-in-groove tryptophan interaction (Kielkopf at al., 2001). The Prp2 also creates a stable complex with Bpb1, a fission yeast homologue of *hs*SF1/BBP/*sc*Msl5 (Huang et al., 2002) and interacts in the yeast two hybrid assay, with Uap2p transcription factor, the homologue of human Tat-SF1 protein (McKinney et al., 1997) and Prp10 splicing factor (Gozani et al., 1998), the homologue of human SAP 155 (Habara et al., 1998).

Deletion of either RRM1 or RRM2 domain independently of each other renders cell lethality, implying the essential function of these domains (Webb et al., 2005a). Based on homology with human $U2AF^{65}$ the RRM1 and RRM2 domains were implicated in binding to Py tract of target introns (Zamore and Green, 1989). However in *S. pombe*, the presence of pyrimidine rich sequence is not a prerequisite for Prp2-dependent splicing, because even the splicing of introns without the Py tract or introns with deleted Py tract required the fully active Prp2 (Romfo et al., 1999, Sridharan and Singh, 2007). Furthermore, Sridharan and Singh (2007) performed experiments with conditional mutant of RRM2 (C387Y) showing that splicing of certain introns does not require the

same activity of Prp2 as the splicing of the others. They showed that the introns with conventional Py tract positioned between the branch site and 3' splice site, as well as the introns lacking the Py sequence, were fully dependent upon the Prp2 activity, whereas the introns with long Py tract upstream of the branch site were not. Interestingly, Prp2-independent introns became Prp2-dependend when the upstream Py tract was deleted, pointing to the possible existence of two independent mechanism for splice site recognition. Furthermore, the mutations D307N and A383Q in RRM2, as well as some point mutations in $U2AF^{SM}$ and Bpb1 proteins caused exon skiping of an artificial, otherwise included exon in *S. pombe* (Haraguchi et al., 2007). This happens in the transcription-dependent manner, showing that Prp2 and the U2AF/Bpb1 complex are important for correct co-transcriptional splice site selection.

The deletion of Prp2 ψRRM has the same effect on the cell viability as the deletion of either RRM1 or RRM2, implying possibility that the fission yeast ψRRM is also involved in RNA binding (Banerjee et al., 2004; Webb et al., 2005a), although no effect on RNA binding and splicing of model introns was observed *in vitro* after the deletion of $U2AF^{65}$ ψRRM domain (Banerjee et al., 2004). The role of ψRRM in the pre-mRNA splicing was for a long time discussed. By using the ψRRM conditional point mutants Sridharan and Singh (2007) showed that fully active ψRRM is important for the efficient splicing of many genes. However, at the same time it was fully or partially dispensable for the splicing of certain introns, usually harboring a long Py tract. Furthermore, Banerjee et al. (2004) pointed at the important role of ψRRM in the protein-protein interactions as the deletion of Prp2 ψRRM abolished the interactions with Bpb1 and Prp10.

1.4. COUPLING SPLICING WITH TRANSCRIPTION

There is a longstanding evidence that transcription and splicing are interconnected *in vivo* (reviewed in Kaufer and Potashkin, 2000; Cramer et al., 2001; Ram and Ast, 2007; Das et al. 2007; Allemand et al., 2008; Pirngruber et al., 2009; Lenasi and Barboric, 2010). The first evidence that splicing affects transcription came from the experiments of Brinster et al. (1988) who showed that the presence of promoter proximal intron(s) enhances transcription of assyed gene (see also Furger et al., 2002). Additional experiments revealed that truncation of RNAPII CTD causes defects in splicing, as well as in capping and polyadenylation (McCracken et al., 1997), and that the purified phosphorylated RNAPII is able to activate the splicing reaction *in vitro* (Hirose et al., 1999). Different transcription factors that affect the rate of transcription were reported to regulate splicing. The direct evidence that the rate of transcription regulates alternative splice site choice came from the experiments of De la Mata et al. (2003), who showed that C4 mutation of human RNAPII reduces the RNAPII transcriptional rate and consequently affects the alternative splicing of selected genes. Later Dye et al. (2006) designed an interesting experiment in which the transcribed intron was co-transcriptionally cleaved. They showed that the intron cleavage does not interfere with the efficient splicing, as would be expected, if it have not been coupled to the transcription. Furthermore, there is also extensive evidence that the phosphorylated CTD acts as a scaffold recruiting the splicing factors to the elongating RNAPII (see part 1.4.1. for details).

1.4.1. Recruitment of splicing factors to RNAPII CTD

Different splicing factors are co-transcriptionally recruited to CTD of RNAPII in phosphorylation-dependent manner. The first splicing factor found to interact with CTD was Prp40, the yeast homologue of FPB11 (Morris and Greenleaf, 2000). Then Fong and Zhou (2001) showed that transcription of reporter HIV-1 template is stimulated by co-transcriptional recruitment of different U snRNPs. This recruitment is mediated by TAT-SF1 transcription elongation factor that in turn interacts with key elongation factor P-TEFb, the heterodimer of CDK9 kinase and cyclin T1. The similar interconnection between splicing factors and P-TEFb was revealed by Bres et al. (2005), who showed that splicing associated protein SKIP directly binds and activates P-TEFb factor *in vivo.* Because SKIP was reported to interact with U5 snRNP and U4/U6.U5 tri-snRNP complexes, these experiments provide an evidence for coupling of the later steps of spliceosomal assembly with the regulation of transcriptional elongation. The key role of CDK9 in coupling transcription with splicing is further underlined by the fact the depletion of 7SK snRNP complex results in induced transcription and enhanced exon inclusion (Barboric et al., 2009; Lenasi and Barboric, 2010). This is important because 7SK snRNP is a complex of 7SK snRNA with several proteins that interact with Cdk9 and inhibit its binding to cyclin T1 and as a consequence inhibit the transcriptional elongation (Nguyen et al., 2001; Yang et al., 2001). Later Kameoka et al. (2004) found the CTD associated transcription factors $p54^{nrb}$ and TLS to cross-link with the 5' splice site of the nascent RNA. The cross-linked proteins were part of a large protein complex composed of hyperphosphorylated RNAPII, a number of transcription elongation factors, U1 snRNP

and U2 snRNP proteins, arguing that $p54^{nrb}$ and TLS directly couple 5' splice site selection with the transcription. Also the proteomic analyses of spliceosomal complexes revealed a number of associated transcription factors with known or unknown function in RNAPII transcription (Zhou et al., 2002; Makarova et al., 2004; Deckert et al., 2006; Behzadnia et al., 2007; Bessonov et al., 2008). The expanded list of factors with possible role in coupling transcription and splicing is reviewed in Allemand et al. (2008).

There exists extensive evidence that spliceosome assemble on CTD of RNAPII in a stepwise manner. Gornemann et al. (2005) showed that the cap binding complex (CBC) is necessary for the co-transcriptional spliceosome assembly pointing at the necessity of co-transcriptional capping for spliceosome assembly. Also the individual spliceosomal spliceosomal complexes are added one after the other. The stepwise assembly of spliceosome was studied *in vivo* by chromatin immunopreciptiation of U1 snRNP, U2 snRNP and U5 snRNP complexes (Lacadie and Rosbash, 2005; Gornemann et al., 2005). With this approach Lacadie and Rosbash (2005) showed that in yeasts the recruitment of U2 snRNP and U5 snRNP is dependent upon the U1 snRNP. They also showed that the U1 snRNP is recruited to transcripts lacking the 5' SS as well, suggesting that the pairing of U1 with 5' SS occurs following the U1 snRNP recruitment to the nascent transcript and not *vice versa*. It is plausible that the U2AF subunit of the pre-spliceosomal complex also associates with RNAPII co-transcriptionally. The $U2AF^{65}$ protein was found to immunoprecipitate in a complex with RNAPII, elongation factor SII (TFIIS) and U1, U2 and U4 snRNPs (Robert et al., 2002; Ujvari et al., 2004). Second, Davies et al. (1998) showed that WT1, a human transcription factor, interacts with $U2AF^{65}$ protein *in vitro* and co-

localize with the splicing factors *in vivo*. Interestingly, the association of WT1 with the splicing factors was enhanced in certain isoforms of WT1 compared to the others, revealing additional level of regulation. Das et al. (2007) analyzed proteins that associate with immunopurified human RNAPII. They found more than 100 proteins associated with RNAPII. Beside the transcription factors a number of SR proteins and U1 snRNP proteins were enriched at RNAPII isolate.

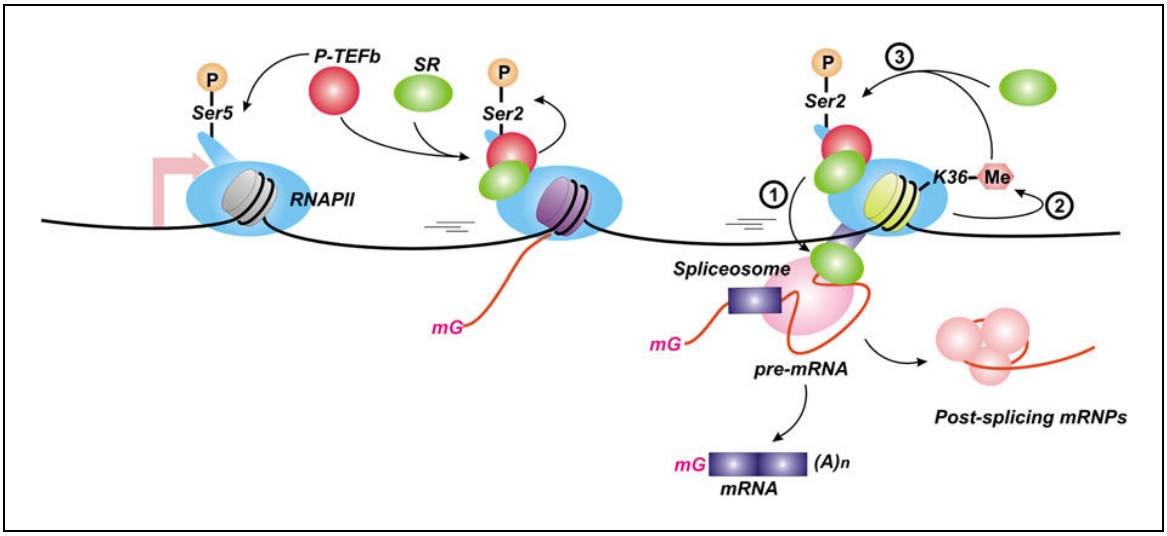

Figure 1.6. A working model for SR protein functions in coupling splicing to RNAPII transcription according to Zhong et al. (2009).

The SR proteins are likely recruited to paused Ser5 phosphorylated RNAPII together with P-TEFb transcription elongation factor concomitant with the Ser2 phosphorylation. When splicing signal emerges out of the RNAPII RNA exit pore the RNAPII associated SR-proteins recognize and bind their target sequences on nascent pre-mRNA (1). As a result H3K36 methylation is increased and RNAPII is transiently paused (2). The RNAPII pausing might help to recruit additional splicing factors to RNAPII and nascent pre-mRNA (3). After successful splicing accomplishment, the RNAPII is released from its paused state to finish pre-MRNA synthesis.

The SR proteins, and also U1 snRNP proteins, were found to interact with RNAPII in RNA-dependent manner, possibly, via U1 snRNP complex. They also used sophisticated *in vitro* transcription assay to show that the SR proteins associated with CTD are required for co-transcriptional splicing of reporter genes, but do not promote their post-transcriptional splicing (Fig. 1.6.). Furthermore, Lin et al. (2008) showed that the depletion of SC35, a well characterized human SR protein, results in reduced Ser2 CTD phosphorylation, defective P-TEFb recruitment and attenuated RNAPII elongation.

1.4.2. Chromatin modifications and co-transcriptional splicing

The CDK9, a kinase component of P-TEFb elongation factor, is responsible for the phosphorylation of Ser2 of RNAPII CTD (see part 1.1.2. for details), but it also phosphorylates some other transcription regulators (Pirngruber et al., 2009). The downstream evens guided by CDK9-dependent phosphorylations result in a complex network of chromatin modifications, including the histon methylations. One possible link between splicing and chromatin modifications is the SWI/SNF chromatin remodeling complex. The catalytic subunit of SWI/SNF complex Brm was reported to interact with splicing factor Sam68 and U1 and U5 snRNAs and regulate the intron incusion/exculsion of several genes in transcription rate-dependent manner (Batsche et al., 2006). Furthermore, the PRP4 kinase, a component of U4/U6.U5 tri-snRNP complex, co-purifies in one complex with BRG1, a catalytic component of SWI/SNF complex and phosphorylates the BRG1 protein *in vitro* (Dellaire et al., 2002). Later Sims et al. (2007) identified a stable complex between CHD1 H3K4me3 methyltransferase and the components of the spliceosome. The physiological relevance of this interaction was

further supported by the fact that the knockdown of CHD1 resulted in reduced levels of H3K4me3 methylation, reduced association of U2 snRNP with chromatin and altered splicing of pre-mRNAs *in vivo*. Another possible link coupling splicing and histon H3 H3K36me3 methylation is the Spt6 transcription elongation factor. It binds to the Ser2 phosphorylated CTD and recruits HYPB/Setd2 histone methyltransferase responsible for H3K36me3 methylation to elongating RNAPII complex (Yoh et al., 2007). The relevance of this finding to the co-transcriptional splicing regulation was also revealed by genome wide comparisons of epigenetic marks relative to various regulatory elements. In this respect it was shown that the 5' ends of exons in humans, flies and *C. elegans* are enriched in the nucleosomes and that this enrichment positively correlate with increased distribution of histon H3 H3K36me3 methylation (Andersson et al., 2009; Schwartz et al., 2009; Hon et al., 2009). The H3K36me3 methylation is also higher on exons relative to introns, and lower on alternatively spliced exons (Andersson et al., 2009; Kolasinska-Zwierz et al., 2009; Schwartz et al., 2009; Hon et al., 2009). The H3K36me3 methylation levels were also positively correlated with gene expression levels. Also some other histon H3 methylations were found to be either enriched or depleted from the exons and also correlated with the exon expression levels (Andersson et al., 2009; Hon et al., 2009).

1.4.3. Transcription regulates alternative splicing in fission yeasts

The majority of *S. pombe* introns are constitutively spliced. However, few reports refer about the existence of several splice variants of a gene (*prp10*, *zas1* and *cds1*) in fission yeasts under the normal growth conditions, i.e. mitotic cell cycle (Habara et al., 1998; Okazaki and Niwa, 2000; Lemaire et al., 2004). However, the alternative splicing of these

genes have not been studied further. The major interest, considering the alternative splicing in fission yeasts, earned the splicing of certain meiotic genes that are specifically spliced during meiosis, but not during the mitotic cell cycle. The first described gene with meiosis-dependent splicing was *mes1* (Kishida et al., 1994; Shimoseki and Shimoda, 2001). It is important to mention that expression of many meiosis-specific genes is transcriptionally regulated. The transcription of genes in meiosis occurs in three successive waves that well correlate with the main events of meiotic differentiation (Mata et al., 2002). The early transcription wave occurs at the same time as the pre-meiotic S phase and recombination events occur. The middle wave correlates with meiosis I and meiosis II and late wave with the spore formation. Employing a systematic analysis Averbeck at al. (2005) showed that 12 out of 96 middle genes tested were specifically spliced during the meiosis I and II, but not in the early stages of meiotic differentiation. The list of genes specifically spliced during meiosis was further extended by Moldon et al. (2008), who assayed for genome-wide Mei4- dependent meiotic splicing, and it now comprises at least 20 different genes (Averbeck et al., 2005; Moldon et al., 2008).

The best studied example of meiosis-dependent splicing is the splicing of *rem1* pre-mRNA (Malapeira et al., 2005; Moldon et al., 2008). The *rem1* gene has two exons interrupted by an intron that harbors stop codon. The unspliced RNA is translated into a short peptide encoded by the first exon of *rem1* gene and this peptide is essential for the proper meiotic recombination (Moldon et al., 2008). However, if spliced, and this happens only during meiosis I (Malapeira et al., 2005), the *rem1* is translated into a

different protein corresponding to the meiotic B-type cyclin. The splicing regulatory element lies within the promoter region of *rem1* gene. This sequence, when placed upstream of a constitutively spliced gene changes its splicing pattern to that of *rem1*. In addition it had been shown that the meiotic splicing of *rem1* requires Mei4 forkhead transcription factor, whereas the intron retention is dependent upon the Fkh2 forkhead transcription factor (Moldon et al., 2008). Furthermore Mei4 is also needed to recruit the active spliceosome to the *rem1* nascent transcript during the transcription. The same type of regulation was observed for some other genes (Moldon et al., 2008). However this is not the only mechanism responsible for the regulated splicing in fission yeasts. For instance, the regulatory elements required for splicing of *mes1* lie within the exon 5 of the gene (Kishida et al., 1994; Shimoseki and Shimoda, 2001) and the regulatory elements required for splicing of another meiotic cyclin *crs1* are located within the 3' UTR (Averbeck et al., 2005). Recently it has been shown thet the inhibition of *crs1* splicing in mitotic cells is regulated by the proximal polyadenylation signal and an element in exon 5, whereas the efficient polyadenylation is the prerequisite for *crs1* splicng in meiosis (McPheeters et al., 2009). Importantly, if polyadenylation status of RNA determines, whether it is spliced or not, the splicing cannot take place until the end of transcription cycle (McPheeters et al., 2009; Moldon and Ayte, 2009).

1.5. PEPTIDYL PROLYL cis/trans ISOMERASES (PPIases) IN THE REGULATION OF SPLICING AND TRANSCRIPTION

The majority of peptidyl bonds in native proteins exist in energetically favourable *trans* conformation (Wang and Heitman, 2005). The exception is the petidyl prolyl bond that can exist in both *cis* and *trans* conformations, because they have similar energy requirements (reviewed in Wang and Heitman, 2005; Shaw PE, 2007; Lu and Zhou, 2007; Lu et al., 2007). In general the transition from *cis* to *trans* conformation requires free energy and is a slow process, unless catalyzed. The peptidyl prolyl bond exists in *cis* conformation in about 5% of cases (Stewart et al., 1990). It is usually found on the surface of proteins, often incorporated within structurally important features, where the *cis* to *trans* isomerisation can have significant impact on the protein structure and function (Pal and Chakrabarti, 1999; Pahlke et al., 2005). The *cis* to *trans* isomerisation is catalyzed by different enzymes that are collectively named peptidyl prolyl *cis/trans* isomerases (PPIases) (see Fig. 1.7., reviewed in Wang and Heitman, 2005; Lu et al., 2007).

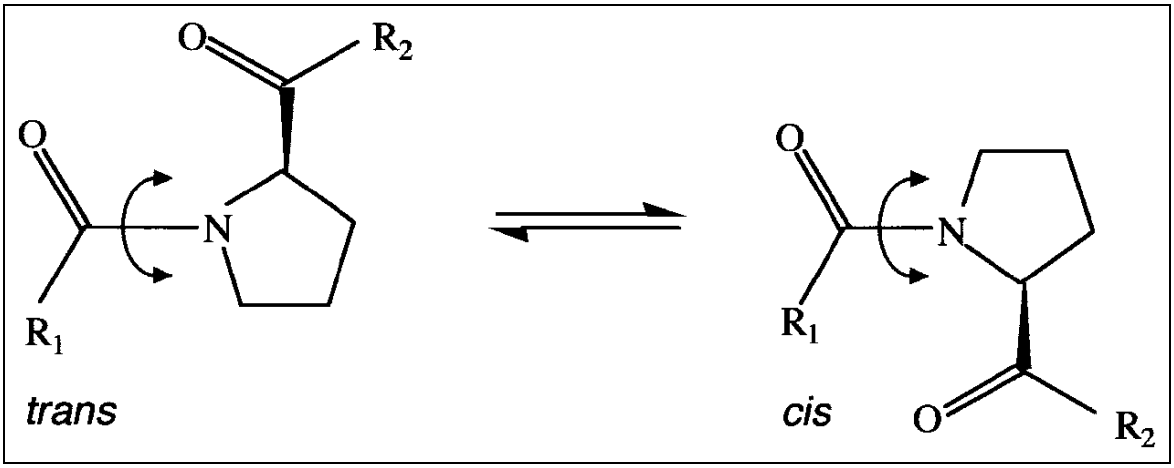

Figure 1.7. Isomerisaton of peptidyl prolyl bonds.

They are further classified into four structurally distinct groups: cyclophilins, FKBPs, parvulins and PTPA. Collectively, the PPIases regulate a number of different cellular processes. For the purpose of the thesis only the functions of PPIases involved in regulation of transcription and splicing will be reviewed.

1.5.1. Regulation of CTD domain phosphorylation by Pin1/Ess1 PPIase.

The best studied PPIase regulating the RNAPII transcription is *hs*Pin1/*sc*Ess1/*sp*Pin1 (reviewed in Lu and Zhou, 2007; Shaw PE, 2007). Pin1 has two domains, the N-terminal WW domain and the C-terminal PPIase domain that are both involved in the substrate recognition and binding. Human Pin1 was originally identified as a mitotic inhibitor (Lu et al., 1996) and subsequent investigations revealed that it has a high number of substrates with different cellular functions (Lu and Zhou, 2007). Later both *hs*Pin1 and *sp*Ess1 were found to interact with phosphorylated CTD of RNAPII *via* the WW domains (Albert et al., 1999; Morris et al., 1999; Wu et al., 2000a; Myers et al., 2001). This interaction became functionally relevant upon the observation that overexpression of *hs*Pin1, as well as the thermosensitive mutation in *ess1* gene, inhibit the RNAPII transcription (Wu et al., 2000a; Xu et al., 2003). The studies on different Pin1/Ess1 substrates revealed that this PPIase specifically recognizes only the phosphorylated pSer-Pro and pThr-Pro residues (Yaffe et al., 1997; Hani et al., 1999). In general the Pin1/Ess1 seems to act on substrates phosphorylated by proline-directed kinases, a subfamily of kinases that specifically recognize Ser and Thr residues followed by proline (Brown et al., 1999; Weiwad et al., 2004). This group of kinases includes cyclin-dependent kinases CDK2, CDK7, CDK8 and CDK9 that are involved in the phosphorylation of RNAPII CTD. The CTD domain

phosphatase Fcp1 was found to suppress the *ess*^{ts} phenotype (Wu et al., 2000a, Kops et al., 2002) and further investigation revealed that the binding of Pin1/Ess1 to CTD inhibits pSer5 dephosphorylation of CTD by Fcp1 (Kops et al., 2002; Xu et al., 2003). Furthermore, the *hs*Pin1 enhances the activity of CDC2/cyclin B kinase towards the CTD resulting in hyperphosphorylated RNAPII (Xu et al., 2003); however, the biological meaning of this RNAPII inhibitory modification remains elusive. The Pin1/Ess1 was shown to be required for proper transcription initiation, termination and splicing, possibly as a result of impaired CTD phosphorylation (Hani et al., 1999; Xu et al., 2003; Wu et al., 2003; Krishnamurthy et al., 2009). Furthermore, Ess1 genetically interacts with CTD kinases Kin28, Ctk1, Srb10 and Bur1 and also with CTD phosphatases Fcp1 and Ssu72, suggesting that it is required for RNAPII transcription in a complex way (Wilcox et al., 2004; Krishnamurthy et al., 2009).

1.5.2. PPIases involved in spliceosome assembly

The PPIases are also frequently found in the spliceosomal complexes purified from different organisms (Zhou et al., 2002; Makarova et al., 2004; Deckert et al., 2006; Chen et al., 2007; Behzadnia et al., 2007; Bessonov et al., 2008). At least seven different PPIases (PPIH, PPIG, PPIE, PPIL1, PPIL2, PPIL3b, PPIL4) were characterized as the spliceosomal components in humans (reviewed in Mesa et al., 2008), but their functions remain poorly undersood.

Probably the best understood is the interaction of PPIH (also known as Snu-Cyp20, USA-Cyp, CypH) with splicing factors *hs*Prp3, *hs*Prp4 and *hs*Prp18. The *hs*Prp3, *hs*Prp4 and

*hs*Prp18 are components of U4/U6 di-snRNP and U4/U6.U5 tri-snRNP complexes that act at the second step of pre-mRNA splicing. The PPIH was found to co-immunoprecipitates with *hs*Prp3 and *hs*Prp4 in both U4/U6 di-snRNP and U4/U6.U5 tri-snRNP complexes (Horowitz et al., 1997, 2002; Teigelkamp et al., 1998) and this interaction was further characterized by the crystal structure of PPIH.U4/U6 snRNP-60K (Reidt et al., 2003). Interesting is the observation of Horowitz et al. (2002), who found that the second step of the splicing reaction is inhibited by cyclosporin A (CsA), a common inhibitor of cyclophilins. Their investigations also showed that CsA inhibits the second transesterification reaction via binding to *hs*Prp18 both *in vivo* and *in vitro*. This suggests that PPIH functions in the regulation of the second step of splicing, as the budding yeast homologue of Prp18 is involved in the stabilisation of the exon-exon interactions in the late spliceosome (Crotti et al., 2007). The fission yeast homologue of PPIH cyclophilin 3 was also reported to create the complex with *sp*Prp3 and *sp*Prp4 (Pemberton et al., 2003), suggesting that the role of this PPIase in the regulation of splicing is evolutionary conserved.

The cyclophilin PPIL1 was also identified as a part of the spliceoseome (Makarova et al., 2004; Deckert et al., 2006). Originally the fission yeast homologue of PPIL1 Cyp2 was identified as an interaction partner of Snw1, the homologue of human transcription co-activator SKIP (Skruzny et al., 2001) and later also the human PPIL1 was found to interact with the SKIP (Makarova et al., 2004; Xu et al., 2006). The PPIL1 was identified also as a part of the larger 35S U5 snRNP.SKIP complex (Makarov et al., 2002) and together with SKIP as a part of the spliceosomal B* complex (Makarova et al., 2004; Deckert et al.,

2006). These observations suggested that the PPIL1 and SKIP functionally interact to couple late spliceosomal rearrangements with transcriptional regulation. However, the direct experimental evidence for this functional coupling is missing.

Another PPIase identified as a part of spliceosomal complexes is the PPIE (also known as Cyp33 and CypE) (Zhou et al., 2002). It is inhibited by CsA and harbours a functional N-terminal, RNA-binding domain (Mi et al., 1996), nevertheless its function is unknown.

Another cyclophilin associated with the spliceosome is PPIG (also known as SR-Cyp, CARS-Cyp, CYPG) (Bessonov et al., 2008). The PPIG has an N-terminal PPIase domain and a long C-terminal RS domain. It was originally identified as an interacting partner of Clk/Sty, the SR protein-specific kinase (Nestel et al., 1996). Bourquin et al. (1997) reported that PPIG interacts with the phosphorylated CTD and that the RS domain of PPIG is required for this interaction. This suggests that the RS domain of PPIG might be important for the coupling of splicing to RNAPII transcription. Cyclophilins with the RS domain similar to PPIG were identified in several organisms: the CypRS64, CypRS92 and AtCyp59 in *A. thaliana* (Lorkovic et al., 2004; Gullerova et al., 2006), the Kin241 in *Paramecium tetraurelia* (Krzywicka et al., 2001), the matrin-cyp in rat (Mortillaro et al., 1998) and the moca-cyp in *Drosophila* (Cavarec et al., 2002). The plant Cyp64 and Cyp92 were shown to interact with plant SR proteins SCL28, SCL30, SRp30 and SRp34 and with the core spliceosomal proteins U1-70K and U11-35K. Interestingly, the interactions with SRp30 and SRp34, the plant homologues of ASF/SF2 splicing factor, were phosphorylation-dependent (Lorkovic et al., 2004). The AtCyp59 was also shown to

interact with a plethora of plant SR proteins (SCL-28,SCL30, SCL30a, SCL33, SC35, SRp30, SRp34,RSZp21, RSZ33, RSp31, RSp40) in RS domain-dependent manner and was also shown to cross-link with the RNA *in vitro* (Gullerova et al., 2006). Furthermore, the authors showed that AtCyp59 interacts with RNAPII CTD *in vitro* and that the ectopic expression of AtCyp59 in plant cells reduces CTD phosphorylation *in vivo*. The AtCyp59 has a distinct domain structure composed of four domains: an N-terminal PPIase, a middle RRM followed by a zink knuckle and a C-terminal RS-like domain. The fission yeast homologue of AtCyp59 the Rct1 (also known as SpCyp6) has a similar domain structure, but it lacks the Zn knucke. Rct1 is an essential protein that interacts with RNAPII CTD *in vitro*, associates with transcriptionally active chromatin *in vivo* and modulates the CTD phosphorylation status in dosage-dependent manner (Gullerova et al., 2007). The heterozygous *rct1/Δrct1* cells were shown to harbour increased Ser2 and Ser5 phosphorylation of CTD, whereas the ectopic overexpression of Rct1 resulted in the hypophosphorylation of the CTD. The *in vitro* experiments showed that Rct1 interacts with CTD Ser2-specific kinases Cdk9 and Lsk1, but not with Ser5-specific kinases Mcs6 and Srb10 and CTD-specific phosphatases Fcp1, Scp1 and Ssu72 (Skrahina T, 2009). Furthermore, the Rct1 interactions with Cdk9 and CTD increase the activity of Cdk9 towards the CTD in a way that specifically requires PPIase domain of Rct1 (Skrahina T, 2009). Although it is plausible that Rct1 acts to couple splicing with the Cdk9 and/or Lsk1-dependent CTD phosphorylation the experimental evidence for this model is missing. Alltogether, the above data suggest that the RS domain PPIases might be important contributors to the coupling of transcription with the spliceosomal assembly.

1.6. AIMS OF THE THESIS

The experimental evidence suggests that the RS domain PPIases might act to couple different steps of spliceosomal assembly with the regulation of RNAPII-mediated transcription (see part 1.5.2. for details). The previous investigations revealed that Rct1 interacts with the Ser2-specific CTD kinases Cdk9 and Lsk1 and regulates RNAPII phosphorylation and transcription in a dosage-dependent manner (Gullerova et al., 2007; Skrahina T, 2009). In this thesis I investigated a possibility that Rct1 acts to couple the pre-mRNA processing with the RNAPII transcription. First I have focused on identification of splicing related proteins interacting with Rct1. For this purpose I performed yeast two hybrid screen with Rct1 protein as a bait and *in vitro* pull down experiments with selected splicing related proteins. The identified interactions were characterized further in the pull down experiments with different deletion mutants of participating proteins. The obtained data suggested that the Rct1 RS domain is important for the interactions with the splicing related factors and therefore I prepared *rct1-HA* and *rct1 ΔRS* strains to study the importance of RS domain in coupling the splicing with transcription. The effect of Rct1 RS domain deletion on the dynamics of RNAPII transcription and splicing was further studied by chromatin immunoprecipitation and RT-PCRs.

2. MATERIALS AND METHODS

2.1. FISSION YEAST STRAINS AND HANDLING OF CELLS

2.1.1. Growing fission yeast cells

The fission yeast strains used in this study are listed in Table 2.1.. They were grown either in rich YES medium or minimal EMM medium (see Tab. 2.3.) at 30-32°C, if not stated otherwise. If needed antibiotics and drugs were supplemented as follows: 155 µg/ml geneticin (GE), 100 µg/ml ClonNat, 100 µg/ml hygromycin (H). To repress expression from *nmt1*$^+$ promoter thiamine was used at 5 µg/ml concentration.

Table. 2.1. Fission yeast strains used in this study.

haploid strains (n)		
name	**genotype**	**source**
wt (wild type)	***h*$^+$** *ade6$^+$, leu1$^+$, ura4$^+$, lys1$^+$, his7$^+$*	Leupold
12155	***h*$^-$** *pat1-114, ade6-M216*	kind gift J. Gregan
12156	***h*$^-$** *pat1-114, ade6-M210*	kind gift J. Gregan
11339	***h*$^+$** *leu1, ura4, lys1, his7, ade6-M210*	kind gift J. Gregan
Δrct1 + pMG1	***h*$^-$** *Δrct1::natMX, ura4-27 (pMG1)*	Gullerova et al. 2007
Δrct1 + pMG2	***h*$^+$** *Δrct1::natMX, ade6-704, ura4-27, (pMG2)*	Skrahina T, 2009
Δrct1 + pMG3	***h*$^+$** *Δrct1::natMX, ade6-704, ura4-27, (pMG3)*	Skrahina T, 2009
Δrct1 + pMG4	***h*$^+$** *Δrct1::natMX, (pMG4)*	Skrahina T, 2009
Δrct1 + pMG5	***h*$^+$** *Δrct1::natMX, ade6-704, leu1-32, ura4-27, (pMG5)*	Skrahina T, 2009
Δrct1 + pMG6	***h*$^+$** *Δrct1::natMX, ade6-704, ura4-27, (pMG6)*	Skrahina T, 2009
Δrct1 + pMG4R3	***h*$^+$** *Δrct1::natMX, ade6-704, (pMG4R3)*	Skrahina T, 2009
rct1-HA	***h*$^+$** *rct1::HA::kanMX*	this study
rct1 ΔRS-HA	***h*$^+$** *rct1-ΔRS::HA::kanMX*	this study
tsn1-GFP	***h*$^+$** *tsn1::GFP::kanMX*	this study
tsn1-HA	***h*$^+$** *tsn1::HA::kanMX*	this study
Δtsn1(nat)	***h*$^+$** *Δtsn1::natMX*	this study
Δtsn1(nat) + pMG	***h*$^+$** *Δtsn1::natMX (pMG)*	this study

Δtsn1(nat) + pMG1	***h*$^+$** *Δtsn1::natMX (pMG1)*	this study
Δtsn1(nat) + pMGT	***h*$^+$** *Δtsn1::natMX (pMGT)*	this study
Δtsn1(kan)	***h*$^+$** *Δtsn1::kanMX*	this study
srp1-FLAG	***h*$^+$** *srp1::FLAG::hphMX*	this study
srp2-FLAG	***h*$^+$** *srp2::FLAG::hphMX*	this study
srp1-tdTomato	***h*$^+$** *srp1::tdTomato::hphMX*	this study
srp1-FLAG rct1-HA	***h*$^+$** *srp1::FLAG::hphMX, rct1::HA::kanMX*	this study
srp1-FLAG rct1 ΔRS-HA	***h*$^+$** *srp1::FLAG::hphMX, rct1-ΔRS::HA::kanMX*	this study
srp2-FLAG rct1-HA	***h*$^+$** *srp2::FLAG::hphMX, rct1::HA::kanMX*	this study
srp2-FLAG rct1 ΔRS-HA	***h*$^+$** *srp2::FLAG::hphMX, rct1-ΔRS::HA::kanMX*	this study
rct1-FLAG	***h*$^+$** *rct1::FLAG::hphMX*	this study
rct1-FLAG + pMGT	***h*$^+$** *rct1::FLAG::hphMX (pMGT)*	this study
diploid strains (2n)		
name	**genotype**	**source**
2n wt (wild type)	***h*$^+$/*h*$^-$** *ade6$^+$/ade6-704, leu1$^+$/leu1-32, ura4$^+$/ura4-27*	Gullerova et al. 2007
2n wt + pMG	***h*$^+$/*h*$^-$** *ade6$^+$/ade6-704, leu1$^+$/leu1-32, ura4$^+$/ura4-27 (pMG)*	this study
2n wt + pMG1	***h*$^+$/*h*$^-$** *ade6$^+$/ade6-704, leu1$^+$/leu1-32, ura4$^+$/ura4-27 (pMG1)*	this study
2n wt + pMGT	***h*$^+$/*h*$^-$** *ade6$^+$/ade6-704, leu1$^+$/leu1-32, ura4$^+$/ura4-27 (pMGT)*	this study
rct1/Δrct1	***h*$^+$/*h*$^-$** *rct1$^+$/rct1:: natMX ade6$^+$/ade6-704, leu1$^+$/leu1-32, ura4$^+$/ura4-27*	Gullerova et al. 2007
rct1/Δrct1 + pMG	***h*$^+$/*h*$^-$** *rct1$^+$/rct1:: natMX ade6$^+$/ade6-704, leu1$^+$/leu1-32, ura4$^+$/ura4-27 (pMG)*	this study
rct1/Δrct1 + pMG1	***h*$^+$/*h*$^-$** *rct1$^+$/rct1:: natMX ade6$^+$/ade6-704, leu1$^+$/leu1-32, ura4$^+$/ura4-27 (pMG1)*	this study
rct1/Δrct1 + pMGT	***h*$^+$/*h*$^-$** *rct1$^+$/rct1:: natMX ade6$^+$/ade6-704, leu1$^+$/leu1-32, ura4$^+$/ura4-27 (pMGT)*	this study
Δtsn1(nat)/Δtsn1(kan)	***h*$^+$/*h*$^-$** *Δtsn1::natMX/Δtsn1::kanMX*	this study
Δtsn1(nat)/Δtsn1(nat)	***h*$^+$/*h*$^-$** *Δtsn1::natMX/Δtsn1::natMX, ade6-M210/ade6-M216*	this study
Δtsn1(nat)/Δtsn1(nat) + pMG	***h*$^+$/*h*$^-$** *Δtsn1::natMX/Δtsn1::natMX, ade6-M210/ade6-M216 (pMG)*	this study
Δtsn1(nat)/Δtsn1(nat) + pMG1	***h*$^+$/*h*$^-$** *Δtsn1::natMX/Δtsn1::natMX, ade6-M210/ade6-M216 (pMG1)*	this study
Δtsn1(nat)/Δtsn1(nat) + pMGT	***h*$^+$/*h*$^-$** *Δtsn1::natMX/Δtsn1::natMX, ade6-M210/ade6-M216 (pMGT)*	this study
tsn1-HA/tsn1-HA	***h*$^+$/*h*$^-$** *tsn1::HA::kanMX/tsn1::HA::kanMX, ade6-M210/ade6-M216*	this study

Δtsn1(nat)/Δtsn1(nat) pat1-114/pat1-114	***h*$^+$*/h*$^+$** *Δtsn1::natMX/Δtsn1::natMX, pat1-114/pat1-114, ade6-M210/ade6-M216*	this study
tsn1-HA/tsn1-HA pat1-114/pat1-114	***h*$^+$*/h*$^+$** *tsn1::HA::kanMX/tsn1::HA::kanMX, pat1-114/pat1-114, ade6-M210/ade6-M216*	this study
rct1-HA/rct1-HA pat1-114/pat1-114	***h*$^+$*/h*$^+$** *rct1::HA::kanMX/rct1::HA::kanMX, pat1-114/ pat1-114, ade6-M210/ade6-M216*	this study

Table. 2.2. Other strains used in this study.

***Saccharomyces cerevisiae* strains**		
name	**genotype**	**source**
AH-109	MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4, gal80, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, MEL1, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ	Clontech
***Escherichia coli* strains**		
name	**genotype**	**source**
XL1-Blue	*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17(r_K-, m_K+), *sup*E44, *rel*A1, *lac*, [F', *proAB*, *lacI*^{q}ZΔM15::Tn10(tet^r)]	lab collection
DH5α^{TM}	ϕ80d*lac*ZΔM15, *recA*1, *endA*1, *gyr*AB, *thi*-1, *hsdR*17(r_K-, m_K+), *supE*44, *relA*1, *deo*R, Δ(*lacZYA-arg*F) U169, *pho*A	lab collection
Top10	F–, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara*, *leu*)7697, *gal*U, *gal*K, *rps*L(strr), *end*A1, *nup*G	lab collection
BL21 (DE3)	F–, *omp*T, *hsd*S_B(r_B-, m_B-), *dcm*, *gal*, λ(DE3)	Kind gift of B. Kusenda
Rosetta (DE3)pLysS	F–, *omp*T, *hsd*S_B(r_B-, m_B-), *dcm*, *gal*, *lacY1*, λ(DE3), pLysSRARE (*argU, argW, ilex, glyT, leuW, proL*) (CmR)	Kind gift of B. Kusenda

Table. 2.3. Media used in this study.

medium	**composition**
LB	0.5% (w/v) yeast extract, 1% (w/v) peptone, 1% (w/v) NaCl
YES	0,5% (w/v) yeast extract, 3% (w/v) D-glucose, 225 mg/l adenine, 225

	mg/l uracil, 225 mg/l L-leucine, 225 mg/l L-lysin, 225 mg/l L-histidine
EMM	0,3% (w/v) potassium hydrogen phthalate, 0,276% (w/v) Na_2HPO_4, 0,5% (w/v) NH_4Cl, 2% (w/v) D-glucose, 0.17% (w/v) $MgCl_2$ $6H_2O$, 0,00148% (w/v) $CaCl_2$ $2H_2O$, 0.1% (w/v) KCl, 0,004% (w/v) Na_2SO4, 0,0001% (w/v) Na pantothenate, 0.001% (w/v) nicotinic acid, 0.001% (w/v) inositol, 0,000001% (w/v) biotin, 0,00005% (w/v) H_3BO_3, 0,00004% (w/v) $MnSO_4$, 0,00004% (w/v) $ZnSO_4$ $7H_2O$, 0,00002% (w/v) $FeCl_3$ $6H_2O$, 0,000004% (w/v) H_2MoO_4 H_2O, 0,00001% (w/v) KI, 0,000004% (w/v) $CuSO_4$ $5H_2O$, 0.0001% (w/v) citric acid, appropriate amino acids and bases used at the concentration 225 mg/l
EMM-NA	as EMM, but 1% (w/v) D-glucose, w/o NH_4Cl, w/o adenine
EMM Ade limiting	as EMM, but 5 mg/l adenine
YPD	1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) D-glucose
SD	0.17% (w/v) YNB, 0.5% (w/v) ammonium sulfate, 2% (w/v) D-glucose, appropriate amino acids and bases
SD+X-Gal	0,67% (w/v) yeast nitrogen base, 2% (w/v) galactose, 1% (w/v) raffinose, 0,7% (w/v) Na_2HPO_4 $7H_2O$, 0,3% (w/v) NaH_2PO_4, 0,008% (w/v) X-Gal, appropriate amino acids and bases

2.1.2. Mating type and ploidy determination

Mating type of desired strain was determined by PCR with two sets of primers (Tab. 2.4.), MM and MT1 that generate 729 bp long product in h^- strains and MP and MT1 that generate 987 bp long product in h^+ strains. Alternatively, mating type was determined based on crosses with established h^- and h^+ strains. To select diploid cells, mating type PCR genotyping and phloxin B selection on YES plates supplemented with 0,02 mg/ml phoxine B were employed. Alternatively, ploidy was checked by sporulation of cells on EMM-NA plate at 25°C for 3-4 days. The spore formation was determined by exposing plates with cells to iodine vapors or microscopically.

Table. 2.4. Primers used for mating type determination in *S. pombe*

primer	**sequence**
MM	TACGTTCAGTAGACGTAGTG

MP	ACGGTAGTCATCGGTCTTCC
MT1	AGAAGAGAGAGTAGTTGAAG

3.1.3 Random spore analysis and tetrad dissection

The haploid fission yeast strains of opposite mating types were allowed to mate and sporulate on EMM-NA plate at 25°C for 3-4 days. Loop of mature spore tetrads was mixed with 900 µl of water and 100 µl of NEE-154 glusulase (PerkinElmer Life Sciences, 10 000 U/ml) and incubated at 30°C for 3-4 hours. After ascus wall digestion the spores were harvested and mixed with 1 ml of 20% ethanol, incubated for 30 minutes at room temperature, harvested again and plated. For tetrad dissection a loop of 2 days old tetrads was spread over the edge of YES plate, tetrads were micromanipulated to desired location and incubated at 20°C overnight. After ascus wall breakage, the tetrades were dissected and allowed to grow for at least 1 week at 32°C.

2.1.4. Non-synchronous sporulation (sporulation assay)

To assay for sporulation efficiency of a diploid strain the fresh cells of desired genotype were diluted with liquid EMM-NA medium to OD_{600} 0,5 and allowed to sporulate at 30°C for 22 hours, if not stated otherwise. After the required period of time they were collected and the percentage of asci generated in the total population of cells was estimated microscopically. The time and temperature were changed, if increased or decreased sporulation efficiency was needed.

2.1.5. Synchronous sporulation of *pat1-114/pat1-114* cells

To obtain synchronous population of *pat1-114/pat1-114* cells at different stages of meiosis the cells of desired genetic background were first diluted in EMM-NA medium to OD_{600} 0,5 and synchronized at 25°C overnight (app. 17 hours). In the morning they were collected and diluted with EMM-NA supplemented with 0,5 g/l NH_4Cl to OD_{600} 0,5 and incubated further at 34°C to induced meiosis. The samples were collected at desired time points after induction of meiosis.

2.1.6. Protoplast fusion

To generate diploid strains by protoplast fusion the required haploid strains were grown up to OD_{600} 0,2-0,3, then 50 ml of each strain were harvested at 4 000 rpm for 3 minutes and the pelleted cells were washed with 10 ml of 0,65 M KCl. Then the cells were harvested again and resuspended in 5 ml of 0,65 M KCl supplemented with 1 mg/ml Novazyme and incubated at 30°C approximately 1 hour, or until protoplasts had appeared (protoplast formation was checked under the microscope). Next, the protoplasts were harvested at 2 500 rpm for 3 minutes, washed with 10 ml of 1,2 M sorbitol and resuspended in 2 ml of 1,2 M sorbitol. Finally, the protoplasts of the strains to be fused were mixed together by pipeting the cells up and down gently, harvested at 2 500 rpm/ 3 min and mixed with 1 ml of 30% PEG 6 000 pre-mixed with 0,1 M $CaCl_2$ in 9 : 1 ratio and incubated for 30 minutes at room temperature. The fused protoplasts were either directly restreaked onto the selective plates supplemented with 1,2 M sorbitol or plated embedded in 1% top-agar.

2.1.7. Fission yeast transformation

For the transformation of fission yeast with plasmid DNA or with DNA replacement cassette 50 ml of fresh overnight culture of OD_{600} 0,3-0,5 were used. The cells were harvested at 3 000 rpm for 2 minutes at the room temperature, washed with 20 ml of water and 1 ml of LiAc-TE buffer [0,1 M lithium acetate (pH 4,9), 10 mM Tris (pH 8), 1 mM EDTA] and finally the pelleted cells were resuspended in 250 µl of LiAc-TE buffer. For transformation 100 µl of cells were mixed with 10 µl of carrier salmon sperm DNA (2 mg/ ml) and 10 µl of the gel-purified DNA replacement cassette (or 1 µl of plasmid DNA). The mixture was incubated at room temperature for 10 minutes, then 260 µl of 40% PEG in LiAc-TE were gently mixed with cells, and the mixture was incubated further at 30°C for 30-60 minutes. Finally, 43 µl of DMSO were added to the mixture, the cells were heat shocked for 5 minutes at 42°C, harvested, diluted with 1 ml of YES media, incubated at 30°C for several hours and plated onto selective plates.

2.2. PREPARATION OF NEW FISSION YEAST STRAINS

2.2.1. Preparation of haploid strains

2.2.1.1. Preparation of *rct1-HA* strain

The *rct1::HA::kanMX* sequence was amplified from pMG1 plasmid with the primers Rct1_KO_F1 and Termin-delPPIase. The replacement cassette was gel purified and transformed into the diploid wild type cells. The transformed cells were plated onto YES plates, replica-plated onto YES+GE plates and re-checked for geneticin resistance. The correct integration of the cassette was verified by PCR with three sets of primers: Spcheck2fw with Seq3REP3XK and Spcypconfw (or kan MX ver F) with Spcheck3rev. The diploids with the cassette correctly integrated were sporulated, the tetrads were dissected and spores were tested for geneticin resistance. The presence of replacement cassette in the geneticin resistant clones was tested by PCR and the PCR products of Spcheck2fw and Seq3REP3XKPCR reaction were then sequenced. The expression of Rct1-HA protein was verified on Western blot with anti-HA antibodies. The selected *rct1-HA* strain was then backcrossed with the haploid wild type strain to obtain the final prototroph strain used in the experiments.

2.2.1.2. Preparation of *rct1 ΔRS-HA* strain

The *rct1-ΔRS::HA::kanMX* sequence was amplified from pMG2 plasmid with the primers Rct1_KO_F1 and Termin-delPPIase. Otherwise the experimental procedure was the same as with *rct1-HA* strain.

2.2.1.3. Preparation of *rct1-FLAG* strain

The *FLAG::hphMX* sequence was amplified from pFA6a-tdTomato-hphMX6 plasmid with the primers Rct1-FLAG-F and Rct1-GFP-rev. The replacement cassette was gel purified and transformed into the haploid wild type cells. The transformed cells were plated onto YES plates and after 20 hours replica-plated onto YES+H plates. Finally, the correct integration of the cassette was verified by PCR with two sets of primers: Rct1-ver-F with MX4/6cassUP-R and MX4/6cassUP with Rct1-ver-R, and subsequent sequencing of the PCR products of the first reaction. Expression of Rct1-FLAG protein was verified on Western blot with anti-FLAG antibodies. The selected *rct1-FLAG* strain was then backcrossed with the 12155 strain to obtain the final prototroph strain used in the experiments.

2.2.1.4. Preparation of *tsn1-HA* strain

The *HA::kanMX* sequence was amplified from pMG-Tsn1 plasmid with the primers Tsn1-promoter-fw2 and Tsn1-terminator-rev. The replacement cassette was gel purified and transformed into the haploid wild type cells. The transformed cells were plated onto YES plates, replica-plated onto YES+GE plates and finally the correct integration of the cassette was verified by PCR with primers Tsn1-UP-fw and Seq3REP3XK and subsequent sequencing of the PCR products. The expression of HA-tagged Tsn1 protein was verified on Western blot with anti-HA antibodies. The selected *tsn1-HA* strain was then backcrossed with the haploid wild type strain to obtain the final prototroph strain used in the experiments.

2.2.1.5. Preparation of *tsn1-GFP* strain

The *GFP::kanMX* sequence was amplified from pSMRG2+GFP5 plasmid with the primers Tsn1-GFP-fw1 and Tsn1-GFP-rev. The replacement cassette was gel purified and transformed into the haploid wild type cells. The transformed cells were plated onto YES plates, replica-plated onto YES+GE plates and finally the correct integration of the cassette was verified by PCR with the primers Tsn1-UP-fw and Seq3REP3XK and subsequent sequencing of PCR products. However no Rct1-GFP protein was detected by fluorescent microscopy and Western blotting.

2.2.1.6. Preparation of *Δtsn1(nat)* strain

The *natMX* sequence was amplified from pAG25 plasmid with the primers Tsn1CNup and Tsn1CNdwn. The replacement cassette was gel purified and transformed into the haploid wild type cells. The transformed cells were plated onto YES plates, replica-plated onto YES+ClonNat plates and finally the correct integration of the cassette was verified by PCR with the primers Tsn1-check1-fw and GFP5-check1-rev and subsequent sequencing of the PCR products. The selected *tsn1-HA* strain was then backcrossed with the haploid wild type strain to obtain the final prototroph strain used in the experiments.

3.2.1.7. Preparation of *Δtsn1(kan)* strain

The *kanMX* sequence was amplified from pMG plasmid with the primers Tsn1-KOup-rev and Tsn1-KOdwn-fw. The replacement cassette was gel purified and transformed into the diploid wild type cells. The transformed cells were plated onto YES plates, replica-plated onto YES+GE plates, sporulated and the tetrads were dissected on YES

plates. Finally, the cells of individual spores were checked for the geneticin resistance and the correct integration of the cassette was verified by PCRs with three sets of primers: Tsn1-UP-fw with Tsn1-DW-rev, Tsn1-UP-fw with KanMX-ver-F and Tsn1-DW-rev with Kan-fw(rev). The selected *Δtsn1(kan)* strain was then backcrossed with the haploid wild type strain to obtain final prototroph strain used in the experiments.

2.2.1.8. Preparation of s*rp1-FLAG* strain

The *FLAG::hphMX* sequence was amplified from pFA6a-hphMX6 plasmid with the primers Srp1-FLAG-F and Srp1-tag-R. The replacement cassette was gel purified and transformed into the haploid wild type cells. The transformed cells were plated onto YES plates, replica-plated onto YES+H plates and finally the correct integration of the cassette was verified by PCR with two sets of primers: Srp1-ver-F1 with MX4/6cassUP-R and MX4/6cassUP with Srp1-ver-R, and subsequent sequencing of the PCR products of the first reaction. The expression of FLAG-tagged Srp1 was verified on Western blot with anti-FLAG antibodies. The selected s*rp1-FLAG* strain was then backcrossed with 12155 strain to obtain the final prototroph strain used in the experiments.

2.2.1.9. Preparation of s*rp1-tdTomato* strain

The *FLAG::hphMX* sequence was amplified from pFA6a-hphMX6 plasmid with the primers Srp1-GFP-F and Srp1-tag-R. The replacement cassette was gel purified and transformed into the haploid wild type cells. The transformed cells were plated onto YES plates, replica-plated onto YES+H plates and the transformants expressing Srp1-

tdTomato were then selected by fluorescent microscopy and on Western blots with anti-dsRed antibodies.

2.2.1.10. Preparation of *srp2-FLAG* strain

The *FLAG::hphMX* sequence was amplified from pFA6a-tdTomato-hphMX6 plasmid with the primers Srp2-FLAG-F and Srp2-tag-R. The replacement cassette was gel purified and transformed into the haploid wild type cells. The transformed cells were plated onto YES plates, replica-plated onto YES+H plates. Finally, the correct integration of the cassette was verified by PCR with two sets of primers: Srp2-ver-F1 with MX4/6cassUP-R and MX4/6cassUP with Srp2-ver-R, and subsequent sequencing of the PCR products of the first reaction. Selected *srp2-FLAG* strain was then backcrossed with the 12155 strain to obtain the final prototroph strain used in the experiments.

2.2.1.11. Preparation of s*rp2-tdTomato* strain

The *tdTomato::hphMX* sequence was amplified from pFA6a-tdTomato-hphMX6 plasmid with the primers Srp2-GFP-F and Srp2-tag-R. The replacement cassette was gel purified and transformed into the haploid wild type cells. The transformed cells were plated onto YES plates, replica-plated onto YES+H plates. However no transformants expressing the tdTomato-tagged Srp2 protein were recovered, as was revealed by fluorescent microscopy and Western blotting.

Table. 2.5. Primers used for the amplification of DNA replacement cassettes. The sequences used for fragment amplification are written in the normal characters. In bold are the sequences used for homologous recombination of the amplified fragments into the *S. pombe* genome and in italic are the sequences encoding the protein tags.

primer	sequence
Tsn1-promoter-fw2	**AATTTACGTGGATTTTTTTTTTTTTTGATAGTTTATTTCTGACATATGCAGTTGTTTATG**ATCTTCGGATACATAAATTG
Tsn1-terminator-rev	**AGCAATTTTATCGGCTCAATTTTAGTCAAGCGTACAGCTGGCAAATAAATTGTTAGCAAT**GGTTAAGGAGTTAGACTCG
Tsn1-GFP-fw1	**GTTTATTTCTGACATATGCAGTTGTTTATGATCTTCGGATACATAAATTGGTT**ATGAGTAAAGGAGAAGAACTT
Tsn1-GFP-rev	**TTTAGTCAAGCGTACAGCTGGCAAATAAATTGTTAGCAAT**GACAAGTTCTTGAAAACAAG
Tsn1CNup	**TCATTCGAATATCAACACTACTCAACAGCATACATTACAGATTAAGTCGAATGAATAAATCAATATTTATTCAGCTACAAGATCAAATTGATAAAGAACA**GATCTGTTTAGCTTGCCTTG
Tsn1CNdwn	**AAATTGTTAGCAATTTAAACCAATTTATGTATCCGAAGATCATAAACAACTGCATATGTCAGAAATAAACTATCAAAAAAAAAAAAAATCCACGTAAATT**AGCTCGTTTTCGACACTGG
Tsn1-KOup-rev	**ACTGAAAACATCATTCGAATATCAACACTACTCAACAGCATACATTACAGATTAAGTCGA**GGTTAAGGAGTTAGACTCG
Tsn1-KOdwn-fw	**AGCAATTTTATCGGCTCAATTTTAGTCAAGCGTACAGCTGGCAAATAAATTGTTAGCAAT**TAATCCTTTCTATTAGTAATGC
Srp1-FLAG-F	**CCAACCCGAAGTGTCAGCTGCTTCAGAGCAACCAGAATCCAACCCTACTACTACAGAGTCTCAA***GATTATAAAGATGACGATGATAAA*TAGCGGATCCCCGGGTTAATTAA
Srp1-tag-R	**ACTGTGTTACGGAATTTTGGAACAAAAGTAAAGGAATATAGACAATTTTTAAAACATGAAAACCTTTTTTTAATTAAGTACAAAAGT**GAATTCGAGCTCGTTTAAAC
Srp1-GFP-F	**CCAACCCGAAGTGTCAGCTGCTTCAGAGCAACCAGAATCCAACCCTACTACTACAGAGTCTCAA**CGGATCCCCGGGTTAATTAA
Srp2-FLAG-F	**AACAGCCTTTGCAAAACCATTCTGATGTAGGTAACGGTAGCGCTGAAGGACAGGTCGCTGCTGAATGG***GATTATAAAGATGACGATGATAAA*TAGCGGATCCCCGGGTTAATTAA
Srp2-GFP-F	**AACAGCCTTTGCAAAACCATTCTGATGTAGGTAACGGTAGCGCTGAAGGACAGGTCGCTGCTGAATGG**CGGATCCCCGGGTTAATTAA
Srp2-tag-R	**GACATATGACAGGAAAATCTAGTAAGCAGACACTAAAAAATAGTTTACCAAAGGAAAAATTTCTATGT**GAATTCGAGCTCGTTTAAAC
Rct1-FLAG-F	**TCACTTACGAGATAAATCTCCTGAACGAAGGTATAGATATGATAGACGTTATAGAGATGATAGATATCGA***GATTATAAAGATGACGATGATAAA*TAGCGGATCCCC

	GGGTTAATTAA
Rct1-GFP-rev	**TGAAGTCTTAATCAATTATTCAACTATGTTTTAGTAATTTCATCAGTCATAAATA ATATTCACAAGAAAGGTACGAATATAATAAAGAT**GAATTCGAGCTCGTTTAAA C

Table. 2.6. Primers used for the verification of correct integration of DNA replacement cassettes.

Name	**sequence**
Tsn1-UP-fw	ATCAACACTACTCAACAGCA
Seq3REP3XK	CTCATCTAAACCACTTTCTAA
Tsn1-check1-fw	TCGTCAATCTGTAAACTCAGTAATATC
GFP5-check1-rev	ATCACCTTCACCCTCTCCAC
Tsn1-DW-rev	CATCATAGGACTGCCAGTTTCATAGC
KanMX-ver-F	GGTTGTTTATGTTCGGATGTGATGTG
Kan-fw-(rev)	GCCATTCTCACCGGATTCAGTCGTC
Srp1-ver-F1	CGTAGTCGTAGTCCTGATGG
MX4/6cassUP-R	GTATTCTGGGCCTCCATGTC
MX4/6cassUP	GACATGGAGGCCCAGAATAC
Srp1-ver-R	TGCCACGAATCAGCACAGTC
Srp2-ver-F1	TCGCCGTCGTTATCGTGATG
Srp2-ver-R	CACATTCCTTTCATTCTTGATCGTAAG
Rct1-ver-F	GCAGAAGCAGAGGCTGTTACA
Rct1-ver-R	CCCTCGAATTTAGTACGACGA

2.2.2. Preparation of diploid strains

2.2.2.1. Preparation of *Δtsn1(nat)/Δtsn1(kan)*, *Δtsn1(nat)/Δtsn1(nat)* and *Δtsn1(kan)/Δtsn1(kan)* strains

To prepare the stable *Δtsn1(nat)/Δtsn1(kan)* diploid strain suitable for the sporulation assay I crossed the haploid *Δtsn1(nat)* strain with the *Δtsn1(kan)* strain of the opposite mating type and selected the desired diploids based on the ClonNat and geneticin

resistance (i.e. on YES+GE+ClonNat plates). To obtain the *Δtsn1(nat)/Δtsn1(nat)* stable diploid strain suitable for plasmid transformations I first crossed the *Δtsn1(nat)* strain with the 12155 and 12156 strains to obtain the *Δtsn1(nat) ade6-M210 pat1*$^{+}$ and *Δtsn1(nat) ade6-M216 pat1*$^{+}$ haploid strains and then crossed these two to obtain the diploids. The desired diploids were selected based on the *ade6* complementation on EMM-A plates. In parallel I prepared the *Δtsn1(kan)/Δtsn1(kan)* diploid strain employing the same approach. The diploid state of prepared strains was further verified by the PCR genotyping, sporulation assay, diploid cell size measurement and Syber Green flow cytometry to quantify the DNA content in the cells.

2.2.2.2. Preparation of *Δtsn1(nat)/Δtsn1(nat) pat1-114/pat1-114* and *tsn1-HA/tsn1-HA pat1-114/pat1-114* strains

To prepare the stable diploid strains suitable for the synchronization in early meiosis I first crossed the *tsn1-HA* and *Δtsn1(nat)* strains with the 12155 and 12156 strains to obtain the *tsn1-HA ade6-M210 pat1-114*, *tsn1-HA ade6-M216 pat1-114*, *Δtsn1(nat) ade6-M210 pat1-114* and *Δtsn1(nat) ade6-M216 pat1-114* haploid strains. These strains were then used to prepare the *Δtsn1(nat)/Δtsn1(nat) pat1-114/pat1-114* and *tsn1-HA/tsn1-HA pat1-114/pat1-114* strains by protoplast fusion of two strains of the same mating type. The diploids were first selected based on the *ade6* complementation on EMM-A plates at 25°C and then checked for the diploid cell size, DNA content by Syber Green based flow cytometry and temperature dependent sporulation phenotype.

2.3. PLASMID PREPARATION

Table. 2.7. General purpose vectors used in this study.

plasmid	general purpose	selection	source
pGBT9	constitutive expression of nuclear GAL4 DNA-binding domain fusion protein from *ADH1* promotor in yeasts	Amp^R, *TRP1*	lab collection
pGAD424	constitutive expression of nuclear GAL4 activation domain fusion protein from *ADH1* promotor in yeasts	Amp^R, *LEU2*	lab collection
pGEX-4T-1	inducible expression of glutathione S-transferase (GST) fusion proteins from *tac* promoter in *E. coli*	Amp^R,	lab collection
pET28a(+)-GST	inducible expression of fusion proteins with C terminal GST tag from T7 promotor in *E. coli*	Kan^R,	lab collection
pMG	repressible overexpression of proteins from $nmt1^+$ promoter in *S. pombe*	*LEU2* Gen^R	lab collection Gullerova et.al 2007

2.3.1. Preparation of pGBT9-Rct1 and pGAD424-Rct1

The $rct1^+$ coding sequence was amplified from *S. pombe* gDNA with primers Rct1-2H-3-PstI and Rct1-2H-5-SmaI. The purified PCR product was digested with PstI and XmaI and the fragments of required size were selected and introduced into pGBT9 or pGAD424 vectors using PstI and XmaI cloning sites.

2.3.2. Preparation of pGBT9-Rct1 ΔRS and pGAD424-Rct1 ΔRS

The *rct1 ΔRS* DNA fragment was amplified from pGBT9-Rct1using primers Rct1-RSdel-2H-3-PstI and Rct1-2H-5-SmaI. The purified PCR product was digested with PstI and XmaI

and the fragments of required size were selected and introduced into pGBT9 or pGAD424 vectors using PstI and XmaI cloning sites.

2.3.3. Preparation of pGBT9-Tsn1, pGAD424-Tsn1, pGBT9-insertTsn1 and pGAD424-insertTsn1

The intronless *tsn1*$^{+}$ coding sequence was amplified from pTN-TH7 library of cDNAs of *S. pombe* using primers Tsn1-Y2H-5 -EcoRI and Tsn1-Y2H-3-BamHI. The purified PCR product was digested with EcoRI and BamHI restriction enzymes and the fragments of required size were selected and introduced into pGBT9 or pGAD424 vectors using EcoRI and BamHI cloning sites. To generate plasmids with a random sequence inserted upstream of *tsn1*$^{+}$, the insert for cloning was amplified with primers Tsn1-Y2H-5-EcoRI-IS and Tsn1-Y2H-3-BamHI from the same template. The inserted sequence was a part of the first primer.

2.3.4. Preparation of pGEX-4T-1-Tsn1 and pGEX-4T-1-Tsn1-HA

The intronless *tsn1*$^{+}$ coding sequence was amplified from pTN-TH7 library of cDNAs of *S. pombe* using primers tsn1 EcoRI-F (or Tsn1-HA-EcoRI-F) and Tsn1-XhoI-R. The purified PCR product was digested with EcoRI and XhoI, the fragments of required size were selected and introduced into vector pGEX-4T-1 using EcoRI and XhoI cloning sites.

2.3.5. Preparation of pMG-Tsn1

The *tsn1*$^{+}$ coding sequence was amplified from pGBT9-Tsn1 plasmid with primers Tsn1-XhoI-ATG and Tsn1-BamHI-HA. The purified PCR product was digested with XhoI and

BamHI, the fragments of required size were selected and introduced into vector pMG using XhoI and BamHI cloning sites.

2.3.6. Preparation of pET28a-Rct1-GST

The *rct1*$^{+}$ coding sequence was amplified from pMG1 plasmid with primers RCT1CGEXUP and RCT1CGEXDW. The purified PCR product was digested with NcoI and SalI, the fragments of required size were selected and introduced into vector pET28a-GST using NcoI and SalI cloning sites.

2.3.7. Preparation of pGEX-4T-1-Srp1, pGEX-4T-1-Srp2, pGEX-4T-1-Prp4, pGEX-4T-1-Ppk15, pGEX-4T-1-Srp1 ΔRS and pGEX-4T-1-Srp2 ΔRS

Corresponding coding sequences were amplified from pTN-TH7 library of cDNAs of *S. pombe* using primers Srp1-BamHI-F and Srp1-XhoI-R for cloning of *srp1*, primers Srp2-BamHI-F and Srp2-XhoI-R for cloning of *srp2*, primers Srp1-BamHI-F and Srp1-ΔRS-XhoI R for cloning of *srp1 ΔRS*, primers Srp2-BamHI-F and Srp2-ΔRS-XhoI-R for cloning of *srp2 ΔRS*, primers Prp4-BamHI-F and Prp4-XhoI-R for cloning of *prp4* and primers Ppk15-BamHI-F and Ppk15-XhoI-R for cloning of *ppk15*. The purified PCR products were digested with BamHI and XhoI, fragments of required size were selected and introduced into vector pGEX-4T-1 using BamHI and XhoI cloning sites.

2.3.8. Preparation of pGEX-4T-1-Dsk1, pGEX-4T-1-Ppk5, pGEX-4T-1-Prp2 and pGEX-4T-1-Prp2 ΔRS

Corresponding coding sequences were amplified from gDNA of *S. pombe* using primers Dsk1-BamHI-F and Dsk1-XhoI-R for cloning of *dsk1*, primers Ppk5-BamHI-F and Ppk5-

XhoI-R for cloning of *ppk5*, primers Prp2-BamHI-F and Prp2-XhoI-R for cloning of *prp2* and primers Prp2-ΔRS-BamHI F and Prp2-XhoI-R for cloning of *prp2 ΔRS*. The purified PCR products were digested with BamHI and XhoI, fragments of required size were selected and introduced into vector pGEX-4T-1 using BamHI and XhoI cloning sites.

Table. 2.8. List of primers used for cloning of inserts into the general purpose plasmids. In bold are the sequences of restriction enzymes used for cloning, in italics are the inserted sequences and in standard upper case are the sequences used for PCR amplification and in lower case are the stop codons.

primer	sequence
Rct1-2H-3-Pst1	GACTAG**CTGCAG**CctaTCGATATCTATCATCTCTATAACGTC T
Rct1-RSdel-2H-3-PstI	ATCT**CTGCAG**ctaACGAGCCACGCTTTGGGA
Rct1-2H-5-Sma1	GACTAG**CCCGGG**ATCTGTACTAATTGAAACTACAGTTGGTG
Tsn1-Y2H-5 -EcoRI	ACTG**GAATTC**AATAAATCAATATTTATTCAGC
Tsn1-Y2H-3-BamHI	ACTG**GGATCC**ctaAACCAATTTATGTATCCG
Tsn1-Y2H-5-EcoRI-IS	ACTG**GAATTC***GTGCATGCTAATGGTTCAGCATTCCTGACTGTGATCAGTAACCTG* AATAAATCAATATTTATTCAGC
Tsn1-EcoRI-F	ATCT**GAATTC**AATAAATCAATATTTATTCAGC
Tsn1-HA-EcoRI-F	ATCT**GAATTC**TATCCGTATGATGTGCCTGACTACGCAAATAAATCAATATTTATT CAGC
Tsn1-XhoI-R	ATCT**CTCGAG**ctaAACCAATTTATGTATCCG
Tsn1-Xho1-ATG	ACTG**CTCGAG**ATGAATAAATCAATATTTATTC
Tsn1-BamHI-HA	ACTG**GGATCC**CTCGACtca*TGCGTAGTCAGGCACATCATACGGATA*AACCAATTT ATGTATCCG
RCT1CGEXUP	ACTG**CCATGG**TGTCTGTACTAATTGAAACTACAGTTG
RCT1CGEXDW	ACTG**GTCGAC**TCGATATCTATCATCTCTATAACG
Rct1-2H-5-Sma1	GACTAG**CCCGGG**ATCTGTACTAATTGAAACTACAGTTGGTG
Rct1-RNA3H-EcoRI	ATCT**GAATTC**ctaACGAGCCACGCTTTGGGA
Tsn1-BamHI-fw	ACTG**GGATCC**TGAATAAATCAATATTTATTCAGC
Tsn1-EcoRI-rev	ACTG**GAATTC**ctaAACCAATTTATGTATCCG
Srp1-BamHI-F	ATCT**GGATCC**AGTCGCAGAAGCCTTCGTAC
Srp1-XhoI-R	ATCT**CTCGA**GctaTTATTGAGACTCTGTAGTAGTAGGG
Srp2-BamHI-F	ATCT**GGATCC**TCGGAGACTAGATTGTTTGTT
Srp2-XhoI-R	ATCT**CTCGA**GctaTTACCATTCAGCAGCGACC
Prp4-BamHI-F	ATCT**GGATCC**AGTGACGATAGATTTGCAGA

Prp4-XhoI-R	ATCT**CTCGAG**ctaTTATTTTTTTATAAAGAAAGGATG
Ppk15-BamHI-F	ATCT**GGATCC**GATTCGGATTCGCCCATT
Ppk15-XhoI-R	ATCT**CTCGAG**ctaTTAGAAAAATTCATCTACATTTTCG
Dsk1-BamHI-F	ATCT**GGATCC**GGAAGTGACGGGTCGAGT
Dsk1-XhoI-R	ATCT**CTCGAG**ctaCTAACGAATTTCAGTAGCCCA
Ppk5-BamHI-F	ATCT**GGATCC**GTGGGGTTAATAAGTACAAGC
Ppk5-XhoI-R	ATCT**CTCGAG**ctaCCATTTCAAACATTTTGAAAG
Prp2-BamHI-F	ATCT**GGATCC**GATTTGTCTTCCAGATTATCATC
Prp2-XhoI-R	ATCT**CTCGAG**ctaTCACCATGCATTAGCTTTATAG
Rct1-Xho1-fw-45	ACTG**CTCGAG**ATGTCTGTACTAATTGAAACTACAGT
Rct1-BamHI-HA-rev-45	ACTG**GGATCC**CTCGACtca*TGCGTAGTCAGGCACATCATACGGATA*TCGATATCT ATCATCTCTATAACG

2.4. YEAST TWO HYBRID SCREEN

To perform yeast two hybrid screen I first amplified pTN-TH7 library in pGAD424 vector (a kind gift of Taro Nakamura) in *E. coli* DH5-α (UltraMAX^{TM} DH5α-FT^{TM} Competent Cells) cells and isolated the amplified plasmid DNA by QIAGENE midi-prep kit according to the instructions of provider. The resulting midi-prep DNA (3,9 µg/µl) was then used for transformation of four different *AH-109 + pGbt9-Rct1* clones in four independent transformation reactions.

The *AH-109 + pGbt9-Rct1* cells used for transformation were first pre-grown overnight in SD-T medium, then diluted in 50 ml of pre-warmed YPDA to OD_{600} 0.5 and then allowed to grow up to the OD_{600} 1,0-1,3. When they reached desired density they were harvested at 3 000 rpm for 5 min, washed with sterile water, mixed with 3 ml of 100 mM Li acetate and incubated for 15 min at 30°C. After the pre-incubation they were collected and the following components were layered over the pellet: 2,4 ml of 50% PEG 3350, 360 µl of 1M Li acetate, 200 µl of ssDNA (5 mg/ml), 36 µl of pTN-TH7 library and 604 µl of water. Finally, the cells were mixed with the components by vortexing, incubated for 30 min at 30°C and heat-shocked for 20 min at 42 °C. After heat-shock the cells were harvested, mixed with 10 ml of water and plated onto SD-TLH plates. The transformation efficiency was estimated after three days of growth at 30°C. The total number of transformants bearing both plasmids was estimated to be 1,834 x 10^6. After six days of growth at 30°C all large colonies (~250 transformants) were streaked onto SD-TLH, SD-TLHA, SD-TLHA + 5 mM 3-AT and SD-TLHA + X-gal plates and checked for phenotypes. After two weeks of

growth additional large colonies and also small, but distinctly white colonies (33 transformants) were selected and checked for phenotypes as above. Finally, 18 clones growing well on SD-TLHA medium were selected and phenotypes of single colonies were re-checked two more times. Finally, the cDNA inserts in pGAD424 vector of selected clones were amplified with pGAD424 5'AD and pGAD424 3'AD primers (Tab. 2.9.), the resulting PCR products were sequenced and obtained sequences were blasted against *S. pombe* genome to identify proteins expressed in selected clones.

Table. 2.9. Oligonucleotides used for insert amplification in the yeast two hybrid

primer	**sequence**
pGAD424 5'AD	CTATTCGATGATGAAGATACCCCACCAAACCCA
pGAD424 3'AD	GTGAACTTGCGGGGTTTTTCAGTATCTACGAT

2.5. PROTEIN ASSAYS

2.5.1. Overexpression and purification of GST-tagged proteins from *E. coli*

E. coli BL21 (DE3) cells transformed with desired plasmids were grown overnight in 10 ml of LB media supplemented with chloramphenicol (40 μg/ml) and ampicilin (100 μg/ml), if pGEX-4T-1 based plasmids were used, or kanamycin (50 μg/ml), if pET28a-GST based plasmids were used. For the GST-protein overexpression the overnight culture was diluted 100 times with fresh medium and incubated at 37°C up to OD_{600} 0,7-1, then IPTG was added to the final concentration of 1 mM and the cells were incubated further at 28°C for another 3-4 hours to allow the protein expression. Finally, the cells were collected for 10 min at 4 000 rpm at 4^0C and stored at -80°C for the GST-protein purification. Expression of the GST-Prp2, GST-Srp2 and GST-Ppk5 was not sufficient in BL21(DE3) strain, therefore *E. coli* Rossetta (DE3) Lys strain was used instead.

The protein extracts for protein purification were prepared as follows: the frozen cells were mixed with 10 ml of lysis buffer [20 mM Tris-HCl (pH 7.5), 1 M NaCl, 0.2 mM EDTA, 1 mM DTT, 1% Triton X-100, 1 tablete of EDTA-free protease inhibitor cocktail (Roche)], sonicated with Bandelin HD 200 Sonoplus sonicator for twice for 15 seconds at 50% duty cycle and cleared by centrifugation at 10 000 rpm for 20 minutes to remove debris. To bind the GST-proteins to the Glutathione Sepharose 4B beads, 400 μl of 50% beads slurry in lysis buffer were mixed with the protein extracts and rolled for 30 minutes at 4°C. The beads with bound GST-protein were washed 3 times with 10 ml of lysis buffer and tree

times with 1 ml of PEB200 buffer on QIAGEN polypropylene columns. The beads with bound GST-proteins were kept in PEB200 buffer at 4°C.

2.5.2. Protein pull down assay

For the protein pull down assay 100 ml of the cell culture overexpressing HA-tagged protein of interest at OD_{600} 0,3-0,5 were collected, mixed with the glass beads and 500-750 µl of PEB400 buffer [50 mM HEPES-KOH (pH 7.9), 400 mM KCl, 1 mM DTT, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 1 mM EDTA (pH 8.0), 1 tablete of EDTA-free protease inhibitor cocktail (Roche)] and sonicated three times with Bandelin HD 200 Sonoplus sonicator for 6 seconds at 50% duty cycle. Prepared cell lysates were left on ice for 10 minutes with occasional vortexing, centrifuged for 15 minutes at 14 000 rpm at 4°C and the supernatants were diluted with PEB0 (same as PEB400, but without KCl), so that KCl concentration of final mixture was 200 mM. For the pull down expriment 300 µl were mixed with the Glutathione Sepharose 4B beads only, 300 µl were mixed with the beads binding the GST fusion proteins and 50 µl of protein extracts were kept as the input sample,. The mixtures were rolled for 2-3 hours at 4°C. The beads with bound proteins were washed four times with PEB200 buffer (same as PEB400, but with 200 mM KCl) and finally the beads were resuspended in 60 µl of Leammlie buffer, denatured for 5 minutes at 95°C and analyzed on Western blotts.

2.5.3. Protein co-imunoprecipitation assay

The procedure for protein co-immunoprecipitation assay was essentially the same as for the protein pull down assay. To prepare the protein extracts 200-250 ml of cell culture with OD_{600} 0,5 were collected, washed with water, mixed with the glass beads and 500 of PEB400 buffer and sonicated four times with Bandelin HD 200 Sonoplus sonicator for 6 seconds at 50% duty cycle. The prepared cell lysates were left on ice for 10 minutes, vortexed occasionally, then centrifuged for 15 minutes at 14 000 rpm at 4°C and finally the supernatants were diluted with PEB0, so that final KCl concentration was 200 mM. The protein concentration in the samples was estimated by BIO-RAD Protein assay kit, and the samples were then diluted so that the final concentration of proteins in PEB200 buffer was the same in all samples. For the immunoprecipitation experiments 50 µl of protein extracts were used as an input sample, 300 µl were mixed with 50 µl of 50% slurry of protein A Sepharose CL-4B beads (GE Healthcare) and 300 µl were mixed with 50 µl of 50% slurry of protein A Sepharose CL-4B (GE Healthcare) beads cross-linked with anti-HA antibody. The mixtures were rolled for 2-3 hours at 4°C, the beads with bound proteins were washed four times with PEB200 buffer and finally, the proteins were washed off the beads with 60 µl of Leammlie buffer, denatured for 5 minutes at 95°C and analyzed on Western blotts.

2.5.4. Preparation of total protein extracts with TCA

To prepare total protein extract of a sample 1ml of a cell culture with an OD_{600} 3 (~ $3x10^7$ cells) was collected, collected cells were washed with water and freezed at -80°C. For the

precipitation of proteins the pellet was mixed with 450 µl of water, 50 µl of 3,5% β-mercaptoethanol in 1,85 M NaOH and incubated for 10 minutes on ice. Then 50 µl of 50% trichloroacetic acid (TCA) were added and the mixture was incubated for 10 minutes on ice. Finally, the proteins were pelleted by centrifugation at 13 000 rpm for 12 minutes at 4°C, washed with 1 ml of acetone, air-dried and mixed with 30 µl of 10% SDS.

2.5.5. Dephosphorylation of proteins with CIP

The protein extracts were prepared in PEB200 buffer as described in 2.6.2. For the protein dephosphorylation 50 µl of the protein extract supplemented with 10 mM $MgCl_2$ were treated with 50 U of CIP phosphatase for 1 hour at 30°C or 37°C, diluted with Leammlie buffer and used for Western blotting. If the concentration of desired protein in PEB200 buffer was not sufficient for visualization on Western blotts, then 500 µl of protein extracts with $MgCl_2$ were treated with 100 U of CIP phosphatase and total proteins were subsequently precipitated with 5% TCA (w/v), dissolved in 20 µl of 10% SDS (w/v), diluted with Leammlie buffer and processed for the Western blotts.

2.5.6. SDS PAGE and Western blotting

If not stated otherwise the proteins were separated using 10% SDS PAGE gels (running buffer: 14,4% glycin (w/v), 3% Tris (w/v), 1% SDS (w/v)) and transferred (transfer buffer: 14,4% glycin (w/v), 3% Tris (w/v)) to PVDF membrane (Millipore) at 400 mA for 45 minutes. After the transfer the membranes were blocked with 5% milk (w/v) in PBS (75 mM NaCl, 3 mM KCl, 4,5 mM Na_2HPO_4, 1,5 mM KH_2PO_4) supplemented with 0,5%

Tritonx-100 (v/v), probed with the primary antibodies (Tab. 2.10.) and horseradish peroxidase conjugated secondary antibodies (Tab. 2.10.), washed with PBST(PBS with 0,5% TritonX-100 (v/v)) and finally washed with PBS. The membranes were then developed with the chemiluminiscence kit (GE Helthcare) and exposed to the Kodak Biomax MR films. If re-probing of membrane was necessary, the membranes were stripped off the antibodies for 30 minutes at 70°C in a stripping buffer [62,5 mM Tris-Cl (6,8), 2% SDS (w/v), 100 mM β-mercapthoethanol] and re-probed with new antibodies as described above.

Table. 2.10. **Primary and secondary antibodies used for Western blotting.**

name	type	source	dilution
primary antibodies			
anti-HA	rat monoclonal IgG	Roche	1:5 000
anti-FLAG	mouse monoclonal IgG	Sigma	1:10 000
anti-RNAPII CTD (8WG16)	mouse monoclonal IgM	Covance	1:1 000
anti-RNAPII CTD (4H8)	mouse monoclonal IgG	Millipore	1:1 000
anti-RNAPII CTD (H5)	mouse monoclonal IgM	Covance	1:1 000
anti-RNAPII CTD (H14)	mouse monoclonal IgM	Millipore	1:1 000
anti- tubulin	mouse monoclonal IgG	Sigma	1:1 000
anti-PSTAIR (or anti-Cdc2)	mouse monoclonal IgG	Abcam	1:1 000
anti-Rct1	rabbit polyclonal	Gullerova et al., 2007	1:1 000
anti-GFP	mouse monoclonal IgG	Roche	1:1 000
anti-dsRed	mouse monoclonal IgG	Clontech	1:500
secondary antibodies			
anti-rat	rabbit polyclonal IgG	Sigma	1:10 000
anti-mouse	goat polyclonal IgG	BioRad	1:10 000
anti-mouse	goat polyclonal IgM	Biosource	1:10 000
anti-rabbit	goat polyclonal IG	Sigma	1:10 000

2.6. CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

2.6.1. Growing the cells and fixation

Cells for ChIP assay were grown overnight in YES medium until they reached OD_{600} 0,5. For cell culture fixation 1/10 of the culture volume of the formaldehyde solution [11% Formaldehyde (v/v), 100 mM NaCl, 1 mM EDTA-Na, pH 8, 0.5 mM EGTA-Na, 50 mM Tris-Cl, pH 8] was added to the culture. The resulting mixture was incubated for 10 minutes at room temperature with constant shaking and then the formaldehyde cross-linking reaction was quenched by the addition of 1M glycine to the final concentration of 125 mM, followed by 10 minutes incubation at room temperature with constant shaking. Finally, the cells were collected by centrifugation at 3 000 rpm for 2 minutes, washed 3 times with 1 ml of ice cold ChIP buffer 1 [50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA (pH 7.5), 1% Triton X-100 (v/v), 0.1% Sodium deoxycholate (w/v)] and frozen at –80°C until use.

2.6.2. Preparation of whole cell extracts and protein immunoprecipitation

For the preparation of whole cell extracts the cells corresponding to 50 ml of fixed and quenched culture were mixed with ~ 50 µl of glass beads in 500 µl of ChIP buffer 1 and sonicated on ice four times with the Bandelin HD 200 Sonoplus sonicator for 6 seconds at 50% duty cycle. To remove debris from the extracts the suspension was centrifugated at 14 000 rpm for 15 minutes at 4°C. The supernatant was then mixed with small aliquot of protein A – Sepharose beads in 20 : 1 ratio and incubated on a rotating wheel for 1 hour at 4°C. After the incubation the beads were pelleted and the pre-cleared cell extracts

were used for the protein immunoprecipitation as follow: 90 µl of cell extracts were frozen for precipitation of input DNA, 200 µl of cell extracts were mixed with desired antibody for protein immunoprecipitation and with protein A - Sepharose beads in 20 : 1 ratio (pA+X) and 200 µl of cell extracts were mixed with beads only and were used as a negative control for unspecific DNA preciptitation (pA). For the RNAPII and FLAG epitope immuneprecipitations 5 µl of the purified anti-RNAPII CTD (4H8) (Millipore) antibodies and 3 µl of anti-FLAG antibodies (Sigma) were added directly to the immunoprecipitation reaction. For the HA epitope immunoprecipitation the beads were first incubated with 1 ml of 12CA5 hybridoma lysates overnight at 4°C to bind the anti-HA antibodies and then the beads were used for the chromatin immunoprecipitation. The mixtures of the beads with the cell extracts were incubated on a rotating wheel for 2 hours at 4°C and then the beads with bound protein complexes were collected and washed with 1 ml of ice-cold ChIP buffer 1 for 10 minutes under the same conditions. Subsequently, the beads were once washed with 1ml of ice cold ChIP buffer 1, ChIP buffer 2 (50 mM HEPES-KOH (pH 7.5), 500 mM NaCl, 1 mM EDTA [10 mM Tris, 1 mM EDTA, pH 7.5), 1% Triton X-100 (v/v), 0.1% Sodium deoxycholate (w/v)], ChIP buffer 3 [10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, pH 7.5, 0.5% Nonidet P-40 (v/v), 0.5% Sodium deoxycholate (w/v)] and finally with TE buffer (pH 7,5). To remove RNA from the mixture, 100 µl of TE buffer containing 10 µg RNase A were added to the beads and the reaction was allowed to proceed for 15 minutes at 37°C. The input sample was treated similarly, but 400 µl of TE buffer with RNase A were added to 90 µl of cell extracts.

2.6.3. Purification of the co-immunoprecipitated DNA

To release the DNA from the cross-linked complexes 2,5 µl of 10% SDS and 2 µl of Proteinase K (600 mAnson-U/ml, Merck) were added to pA+X and pA samples. The protein digestion was performed for 8 hours at 37°C and was followed by decross-linking of samples for 8 hour at 65°C. The released DNA was then purified by the extraction with 10 µl of 3 M sodium acetate (pH 5,2) and 100 µl of phenol/chloroform/isoamylalcohol. The phases were separated by centrifugation at 14 000 rpm for 15 minutes at room temperature. The DNA was precipitated from 130 µl of the supernatant by 40 µg of glycogen and 250 µl of 100% ethanol at -20°C overnight. The precipitated DNA was pelleted for 15 minutes at 13 000 rpm at room temperature, washed with 70% ethanol and dissolved in 150 µl of water.

The input sample was treated slightly differently. To release the DNA from the cross-linked complexes, 10 µl of 10% SDS and 2 µl of Proteinase K were added to 90 µl of the input sample. The protein digestion and decross-linking were performed in the same way as above. The released DNA was purified by the extraction with 40 µl of 3 M sodium acetate (pH 5,2) and 400 µl of phenol/chloroform/isoamyl alcohol and the DNA was precipitated from 450 µl of the supernatant by 40 µg of glycogen and 1 ml of 100% ethanol at -20°C overnight. Finally, the precipitated DNA was dissolved in 150 µl of water.

2.6.4. Real time PCR quantification of the co-imunoprecipitated DNA

The relative amount of precipitated DNA was estimated by a quantitative real-time PCR (qRT-PCR) using 10 µl of LightCycler 480 SYBR Green I master kit, 2 µl of immunoprecipitated DNA, 2 µl of 2 µM primer mixture and 6 µl of water per reaction. The PCR conditions were as follow: 1. 95°C/10 minutes, 2. 95°C/15s, 3. 45°C/30s, 4. 68°C/10s, 5. 95°C/15sec, 6. melting curve: 50°C-->95°C/10 minutes, repeating 40× steps 2 to 4. The PCR reaction was performed with Eppendorf Realplex Mastercycler and the data were analyzed using the Realplex 2.0 or 2.2 software. The primers used for DNA amplification are listed in Tab. 2.11.. The relative amounts of immunoprecipitated DNA were expressed as a function of the Ct values and the primer efficiency was calculated according to the following formula: the relative amount of DNA = $E^{-(Ct1-Ct2)}$, where the Ct1 stands for threshold Ct value of the immunoprecipitated sample and the Ct2 for threshold Ct value of the input DNA. The primer efficiency (E) of individual primer pairs was estimated based on five serial dilutions of the genomic DNA according to the following formula: E = $10^{-(1/S)}$, where S stands for the slope of a standard curve plotting the $\log_{10}$(DNA amount) against the corresponding Ct values. (Pfaffl MW, 2001)

Table. 2.11. Primer pairs used in quantitative real-time PCRs.

position	primer	sequence	Size of product
primer probes in rDNA region			
C	rRNA22f	AGTAAGGATTGACAGATTGAGAG	88 bp
	rRNA22r	AAGCAGACAAATCACTCCAC	
primer probes in the *dis2* region			
D0	D1fw	ACATTCCATCCATCAAATTT	92 bp

	D1rev	GAACCCTAGACTTTTCTCTTCA	
D1	D2fw	AGTGATTATAGAAACTAGGAATATAGG	91 bp
	D2rev	TAGGTAGTTGAATGGTTGGG	
D2	D3fw	CTTCATAAGTGTCAAAAGCG	90 bp
	D3rev	TCTAACAAGCGGTCGATAAT	
D3	D4fw	GATTCTTGGTCTCATTTGTG	92 bp
	D4rev	CACGATATATGCCAATATGC	
D4	D4/5 fw	TTTATTAGTCAACCTATCCTTCTAG	90 bp
	D4/5 rev	CCGTACTCAAAAAGTCGAA	
D5	D52fw	TATAAAATCAAGTACCCCGAA	96 bp
	D52 rev	CTTACATTCATCGTAAAATCCA	
D6	D5/6-3F	CTTTCGCCAGATTTGAACTC	88 bp
	D5/6-3R	CACAGAGTAACCCAGTATCA	
D7	D6fw	AATCTGACGCTTAAAAGACG	87 bp
	D6rev	GTTAAGCACTCTCTCGAAGATA	
primer probes in the *rho1* region			
R1	R1fw	TTACACTCTGTACAGGCTCA	103 bp
	R1rev	AGACGCTACTACACTTCACT	
R2	R2fw	GTCTAGAGCAGGAGAAAGTA	90 bp
	R2rev	TTTACCACATGCACCATCTC	
R3	R3-2 fw	TGGGCCTGTTTTAAATTGGC	92 bp
	R3-2 rev	CTTGAGGCAATGAACATGCT	
R4	R4fw	CACGAGTCTGTGTCATTGTT	95 bp
	R4rev	AAACAGTGGGAACATAGACC	
R5	R5fw	AAGATTACGACCGTCTACGT	93 bp
	R5rev	CATTGTCAAGAGAATCGGGA	
R6	R6fw	CAGTCGAAGCACATTCAACA	89 bp
	R6rev	ACAATGCAAAACTCCCACCA	
primer probes in the *osm1* region			
O1	O1fw	AGACAGCATCTAACCACGAA	90 bp
	O1rev	AGGCATCGAAGAGAAAAGCA	
O2	O2fw	TACATGGACTTTTAGGCGTC	89 bp
	O2rev	AGCAGTGGACATGTTGAATC	
O3	O3fw	CGACACGTATGGATCTTGTT	98 bp
	O3rev	ACCACAATATGCCAGACGTA	
O4	O4fw	TGGAGGAAATAGCGTCAAAG	85 bp
	O4rev	TACACTGTCGGAAACATGCT	
O5	O5-2fw	TGGAATACATGGACTTGAGC	91 bp
	O5-2rev	TCACTGTAATCGGCAGCATA	

O6	O6fw	ACACCGAAGGAATCCTCTTT	89 bp
	O6rev	TGCTCTATCGAACAAGAAGC	

2.7. RNA ASSAYS

2.7.1. RNA isolation with hot phenol

For RNA sample preparation the cells equivalent to 1 ml of culture with OD_{600} 3 (~ 3×10^7 cells) were collected at 3 000 rpm for 2 minutes at 4°C, washed with ice cold water and frozen at -80°C. For the RNA extraction the pellets were defrosted on ice, mixed with 400 µ1 of AE buffer (50 mM NaOAc, 10 mM EDTA, pH 5.0), 40 µl of 10% SDS and 440 µl of AE buffered phenol, vortexed briefly, heated for 5 min at 65°C, incubated on dry ice until phenol crystallized (half of the volume should be crystals) and then the phases were separated at 13 000 rpm for 2 min at room temperature. To remove any residual traces of DNA the upper aqueous phase was treated with 5 U of RQ1 DNase (Promega) for 30 minutes at 37 °C, then mixed with 1 volume of phenol : chlorophorm (1:1), vortexed briefly and the phases were separated at 12 000 rpm for 5 min at room temperature. To precipitate the RNA the upper aqueous phase was mixed with $1/10^{th}$ of volume of 3 M NaOAc (pH 5,3) and 1 volume of isopropanol, incubated at -20 °C for 10 min and centrifuged at 14 000 rpm for 10 min at 4°C. The RNA pellet was washed with 500 µl of ice cold 70% ethanol, air-dried for 10 min and dissolved in 20 µl of RNase-free water.

2.7.2. Quantitative and semiquantitative RT-PCRs

For the reverse transcription 1 µg of RNA was diluted in 5 µl of RNAse-free water, the sample was denatured at 70 °C for 5 minutes, centrifuged briefly and placed on ice. The

reaction components (reverse transcription system, Promega) were added to RNA while on ice as follows: 4 µl of $MgCl_2$ (25 mM), 2 µl of reverse transcription buffer (10×), 2 µl of dNTPs mixture (10 mM), 0,5 µl of recombinant RNasin ribonuclese inhibitor (40U/µl), 0,5 µl of AMV Reverse Transcriptase (20U/µl), 1 µl of Oligo$(dT)_{15}$ primer (0,5 µg/µl) and 5 µl of RNAse-free water. The reverse transcription was carried out at 42°C for 15 min, stopped at 95°C for 5 min and finally the cDNA was incubated on ice for at least 5 min. For the quantitative amplification of RT-PCR product 1/100th volume of reaction was used for the real-time PCR amplification of the desired targets with LightCycler 480 SYBR Green I master kit under the same condition as designed for the quantification of ChIP DNA (3.7.4.). For the semiquantitative amplification of the RT-PCR product 1/10th volume of the reaction was used as template in PCR with Dream Taq DNA polymerase (Fermentas) and the PCR products were analysed on ethidium bromide stained 2% agarose gel. The PCR conditions were as follows: 1. 95°C/5 minutes, 2. 92°C/ 30s, 3. 45°C/30s, 4. 72°C/60s, 5. 95°C/10 minutes, repeating steps 2 to 4 26 times with oligos priming at *dis2*$^{+}$ and *osm1*$^{+}$ gene and 25 times with the oligos priming at *rho1*$^{+}$gene. The primer combinations used are listed in the Tab. 2.12..

Table. 2.12. Primer used for semiquantitative amplification of RT-PCR products. The primers were the same as primers used for the quantification of ChIP DNA (Tab. 2.11.), except of *act1* primers, but used in different combinations.

mark	**forward primer**	**reverse primer**
dis2		
1.	D4 fw	D5/6-3 rev
2.	D3 fw	D45 rev
3.	D4/5 fw	D5/6-3 rev
osm1		
1.	O3 fw	O4 rev
2.	O2 fw	O4 rev
3.	O4 fw	O5 rev
rho1		
1.	R3-2 fw	R4 rev
2.	R5 fw	R5 rev
3.	R2 fw	R4 rev
act1		
4.	Act1 ORF fw	Act1 ORF rev
primer name	**sequence**	
Act1 ORF fw	AGCACCCTTGCTTGTTGA	
Act1 ORF rev	TGGGAACAGTGTGGGTAACA	

2.8. IMMUNOSTAINING OF FORMALDEHYDE FIXED CELLS

For the immunostaining the exponentially growing cells were fixed with freshly prepared 38% formaldehyde solution. To prepare the formaldehyde solution 380 mg of paraformaldehyde were dissolved in 700 µl of PEM buffer (100 mM Pipes, 1mM EGTA, 1 mM $MgSO_4$, pH 6.9 with NaOH) mixed with 48 µl of 5M NaOH, incubated at 65°C for 30 minutes, centrifuged at 5 000 rpm for 3 minutes and the supernatant was used further. To fix the cells $1/10^{th}$ of volume to be fixed of formaldehyde was added to the cell culture and then the mixture was incubated at 32°C for 70 minutes. After the fixation the cells were collected at 13 000 rpm for 5 minutes (the same setting were used in the next steps), washed three times with PEM buffer, then resuspended in 1 ml of PEMS buffer (PEM buffer with 1,2 M sorbitol) containing 0,5 mg of zymolyase (Seikagaku corporation) and incubated at 37°C for 1 hour. After the cell wall digestion the cells were washed three times with 1 ml of PEMS buffer, resuspended in 1 ml of PEMT buffer (PEM with 1% Triton), incubated for 2 minutes at room temperature and finally washed three times with PEM buffer and kept in 100 µl of PEM buffer containing 0,1% NaN_3 at 4°C overnight. Before a primary antibody was added the cells were collected and resuspended in 100 µl of PEMBAL buffer (PEM with 1% BSA, 100 mM lysine and 0,1% NaN_3), incubated for 1 hour at room temperature on a rotating wheel, then collected and resuspended in 100 µl of PEMBAL buffer. Finally, either 1 µl of monoclonal rat anti-HA antibodies (Roche), or 1 µl of monoclonal mouse anti-FLAG antibodies (Sigma) was added and the samples were incubated overnight on the rotating wheel wrapped in aluminum foil.Before the addition

of a secondary antibody, the cells were washed three times with 100 µl of PEMBAL, then collected and resuspended in 100 µl of PEMBAL buffer containing either 1 µl of anti-rat Alexa 568 antibodies or 1 µl of anti-mouse Alexa 568 antibodies and incubated overnight on the rotating wheel wrapped in aluminum foil. At the end the cells were washed four times with PEM buffer, resuspended in 100 µl of PEM buffer containing 0,1% NaN_3 and kept at 4°C. The immunostained cells were then examined with the Zeiss Axioscope fluorescent microscope.

2.9. MEASURING DNA CONTENT BY FLOW CYTOMETRY

Cells of interest were fixed with ice-cold 70% ethanol (final concentration of ~ 10^7 cells/ ml) and stored at 4 °C. Before staining 100 µl of fixed cells were washed with 3 ml of 50 mM Na citrate buffer, harvested at 2500 rpm for 3 minutes, mixed with 0,5 ml of 50 mM Na citrate buffer supplemented with 0,1 mg/ml of RNase A and incubated at 37°C for 2 hours. To stain the cells with Sytox Green, 0,5 ml of 50 mM Na citrate buffer containing 2 µM Sytox Green was added at least 30 minutes before measurement. The measurement was performed with FACS Calibur flow cytometer.

3. RESULTS

3.1. IDENTIFICATION OF NEW PROTEINS INTERACTING WITH Rct1

3.1.1. Fission yeast protein Rct1 interacts with Tsn1 protein in yeast two hybrid assay

Fission yeast protein Rct1 is a multidomain cyclophilin with many putative protein targets in cell. Only few proteins involved in the regulation of transcription are known to interact with Rct1 and these interactions are mediated solely by PPIase domain of Rct1 (Gullerova et al., 2007; Skrahina T, 2009). To extend the palette of known proteins interacting with Rct1 I performed yeast two hybrid screen with Rct1 protein as bait. For this purpose I cloned Rct1 coding region into pGBT9 vector and created pGBT9-Rct1 plasmid expressing GAL4-BD-Rct1 fusion protein. I used this plasmid to screen *S. pombe* pTN-TH7 library of mitotic and meiotic cDNAs in pGAD424 vector expressing proteins fused with GAL4-AD (kindly provided by Taro Nakamura). The screen itself was performed in *S. cerevisiae* strain AH-109 that allows selection of positive clones by use of three independent reporter genes: *ADE2*, *HIS3* and *lacZ*. The cells transformed with both plasmids were first screened for positive interactions under low selective conditions (SD-HLT), and later under mild (SD-HLTA) and high (SD-HLTA + 5 mM 3-AT) selective conditions. Employing this approach I was able to select 18 clones from total number of 1,834 x 10^6 transformants that grew well under mild selective conditions (1-18 in Fig. 3.1.A., Tab. 3.1.) and they were further characterized. Only five of them turned blue in X-Gal assay (marked in bold in Tab. 3.1.) and the same five also grew best in the presence of 5 mM 3-AT, a competitive inhibitor of His3 protein (Fig.3.1.A.). To indentify GAL4-AD

fusion proteins in selected clones, the inserts in pGAD424 vector were amplified by PCR with pGAD424 specific primers and sequenced. The obtained sequences were blasted against *S. pombe* genome to identify the expressed genes. The clones expressing proteins with shifted ORF or those with early stop codon were excluded from the analysis

Table 3.1. List of proteins identified in yeast two hybrid screen with full length Rct1 protein.

Table of genes encoded by sequences in pGAD424 vector in the clones selected in the yeast two hybrid screen. The numbers 1-18 correspond to the clones shown in Fig. 3.1.A. Gene names and the assigned protein products are according to *Schizosaccharomyces pombe* GeneDB database (http://old.genedb.org/genedb/pombe/). In bold are the clones that turned blue in X-Gal assay and grew best in the presence of 5 mM 3-AT. In italics are the clones that were excluded from the analysis due to an early stop codon or a frame shift.

clone	gene name	product	coding sequence
1	*SPBC31F10.12*	*predicted RNA-binding protein* Tma20	*early stop codon*
2	-	*non coding sequence*	*early stop codon*
3	*ef1a-c*	translation elongation factor EF-1 alpha Ef1a-c	partial coding sequence
4	-	*non coding sequence*	*early stop codon*
5	*bms1*	GTP-binding protein Bms1	partial coding sequence
6	*get1*	*predicted GET complex subunit* Get1	*not in the correct frame*
7	-	*SQV orphan*	*early stop codon*
8	*SPBP35G2.11c*	transcription related zf-ZZ type zinc finger protein	partial coding sequence
9	-	*non coding sequence*	*early stop codon*
10	***SPBC1347.05c***	**DNAJ domain protein Scj1**	**partial coding sequence**
11	***tsn1***	**translin**	**full length coding sequence**
12	***SPBC1347.05c***	**DNAJ domain protein Scj1**	**partial coding sequence**
13	*rpl13*	*60S ribosomal protein* L13	*not in the correct frame*
14	*idh2*	isocitrate dehydrogenase (NAD^+) subunit 2	partial coding sequence
15	*SPBC1718.04*	predicted glycerol-3-phosphate O-acyltransferase	partial coding sequence
16	***tsn1***	**translin**	**full length coding sequence**
17	*tsn1*	translin	partial coding sequence
18	***SPBP35G2.11c***	**transcription related zf-ZZ type zinc finger protein**	**partial coding sequence**

(marked in italic in Tab. 3.1.). The remaining sequences encoded even different proteins expressed either as the full length proteins or their truncated versions. Among the selected clones I found three times sequence encoding translin (Tsn1), twice in full length (#11 and #16) and once in its truncated version (#17), two times partial sequence encoding part of an uncharacterized transcription related protein SPBP35G2.11c (#8 and #18), two times sequence corresponding to DNAJ domain protein Scj1 (#10 and #12) and sequences encoding parts of translation elongation factor EF-1 alpha (#3), GTP-binding protein Bms1 (#5), isocitrate dehydrogenase Idh2 (#14) and a predicted glycerol-3-phosphate O-acyltransferase SPBC1718.04 (#15). For further characterization I selected translin as the most likely protein interacting with Rct1.

Next, I repeated the yeast two hybrid assay with *de novo* prepared plasmids expressing Tsn1 fusion proteins. For this purpose I cloned the cDNA corresponding to *tsn1*$^{+}$ coding region into pGAD424 and pGBT9 vectors to generate pGAD424-Tsn1 and pGBT9-Tsn1 plasmids. These were used in the combination with pGAD424-Rct1 and pGBT9-Rct1 plasmids in the yeast two hybrid assay under the same conditions as described above. In this reconstituted system GAL4-AD-Tsn1 fusion protein was able to interact with GAL4-DB-Tsn1 protein. This is well consistent with the previous reports showing that translin oligomerises *in vivo* and *in vitro* (VanLoock et al., 2001; Sugiura et al., 2004; Gupta et al., 2008). Surprisingly, no positive interaction between Rct1 and Tsn1 was detected in this assay (Fig.3.1.B.). Interestingly, all translin sequences obtained in the yeast two hybrid screen included the following sequence: CGAATATCAACACTACTCAACAGCATACATTA-CAGATTAAGTCGAATG, a part of the 5' UTR of *tsn1* gene inserted between GAL4-AD

domain and translin coding sequence. This 5' UTR sequence, when translated into the peptide (Arg-Ile-Ser-Thr-Leu-Leu-Asn-Ser-Ile-His-Tyr-Arg-Leu-Ser-Arg-Met), could possibly serve as a hinge between the GAL4-AD domain and translin peptide, spacing the translin into a position more suitable for the interaction with Rct1. Therefore, I decided to clone a random sequence (GTGCATGCTAATGGTTCAGCATTCCTGACTGTGATCAGTAACCTG translated as Val-His-Ala-Asn-Gly-Ser-Ala-Phe-Leu-Thr-Val-Ile-Ser-Asn-Leu) between the GAL4-AD and Tsn1 coding domains in pGAD424-Tsn1 and pGBT9-Tsn1 vectors and repeat the yeast two hybrid assay with the new plasmids. However, the insertion of the random sequence upstream of the *tsn1*$^{+}$ coding region did not recover the interaction between Rct1 and Tsn1 in the yeast two hybrid assay (data not shown). This experiment points to the possibility that the insert sequence identified in the yeast two hybrid screen is essential for the yeast two hybrid interaction and cannot be simply replaced by a random sequence.

The N-terminal PPIase domain of Rct1 has a putative enzymatic activity and likely targets different proteins. To obtain more detailed information on protein targets bound by PPIase domain of Rct1 I deleted the sequence encoding the C-terminal RS domain and cloned the truncated Rct1 coding region into pGBT9 vector generating pGBT9-Rct1 ΔRS plasmid for yeast two hybrid assay. By this approach I aimed to eliminate RS domain-dependent interactions and enrich those mediated by PPIase domain. Surprisingly, when I tested the cells transformed with pGBT9-Rct1 ΔRS plasmid in the combination with empty pGAD424 vector for growth under the selective conditions I observed a robust cell growth on the selective media (Fig. 3.1.C.). Based on this experiment I concluded that the

truncated GAL4-BD-Rct1 ΔRS protein is able to induce transcription of reporter genes by its own in the presence of GAL4-AD protein, although no protein is fused to GAL4-AD domain.

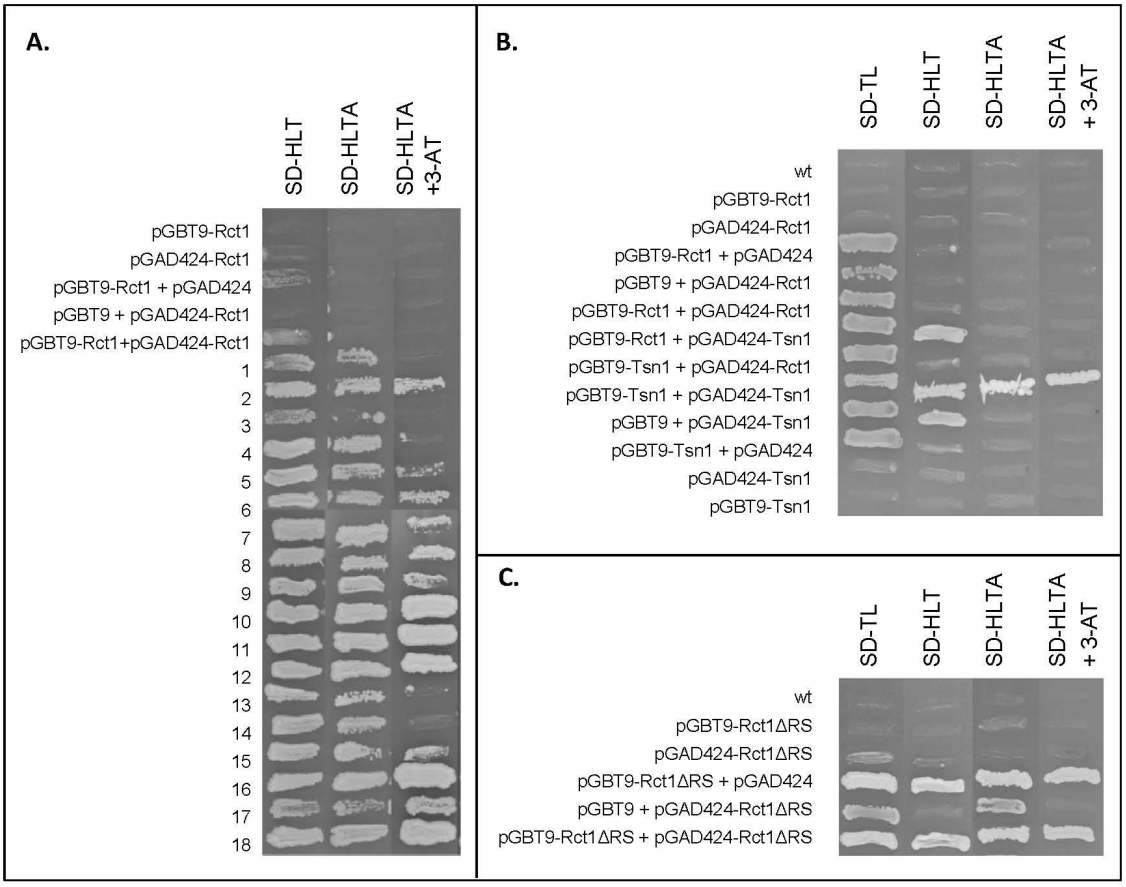

Figure 3.1. Proteins interacting with Rct1 identified in the yeast two hybrid screen.

A. Yeast two hybrid assays with *S. cerevisiae* strain AH109 transformed with different pGBT9- and pGAD424-based plasmids in combinations indicated on the left side of the picture (lanes 1-5) and with clones 1-18 selected in the yeast two hybrid screen (lanes 6-23) grown under the low selective conditions (SD-HLT), mild selective conditions (SD-HLTA) and high selective conditions (SD-HLTA supplemented with 5 mM 3-AT). **B.** Verification of the yeast two hybrid interactions between Rct1 and Tsn1 using the newly created pGAD424-Tsn1 and pGBT9-Tsn1 vectors. The AH109 strain (lane 1) was transformed with different pGBT9 and pGAD424 derivatives in combinations indicated on the left side. **C.** Yeast two hybrid assay pictures showing that pGBT9-Rct1 ΔRS in combination with empty pGAD424 or pGAD424-Rct1 ΔRS plasmid can activate transcription of reporter genes without the partner protein. Again the assays were performed in AH109 strain using the indicated combinations of plasmids.

I also cloned Rct1 ΔRS protein into pGAD424 vector generating pGAD424 Rct1 ΔRS plasmid and used it in the combination with empty pGBT9 vector in the same yeast two hybrid assay. However, the cells expressing GAL4-AD-Rct1 ΔRS and the untagged GAL4-DB protein were not able to grow under the selective conditions (Fig. 3.1.C.). Therefore, I concluded that GAL4-DB domain of GAL4-BD-Rct1 ΔRS protein is essential to target Rct1 ΔRS to the proximity of reporter genes and once nearby the Rct1 ΔRS peptide can activate their transcription as an activator. Importantly, when pGBT9-Rct1 plasmid is used, and full length Rct1 is targeted to chromatin by GAL4-DB domain, the reporter genes are not activated. It is therefore the deletion of RS domain that changes activity of Rct1 protein in *S. cerevisiae* cells and it is possible that the proteins binding or modifying RS domain of Rct1 *in vivo* might also modulate activity of the whole Rct1 protein.

3.1.2. Tsn1 is fission yeast homologue of translin, a highly conserved protein in higher eukaryotes

Translin is referred as a highly conserved protein in higher eukaryotes (Li et al., 2008; Jaendling and McFarlane, 2010) with homologues in plants and animals, but like in case of Rct1 (Gullerova et al., 2007), there is no recognized homologue protein in budding yeasts. To verify this information I compared fission yeast translin protein sequence with sequences of *Homo sapiens*, *Mus musculus*, *Galus galus*, *Drosophila melanogaster*, *Xhenophus laevis* and *Arabidopsis thaliana (*Fig. 3.2.A.). Although there is high similarity between mammalian translins, as shown by 98% similarity between mouse and human translin, the fission yeast translin is not similar to any of the analyzed translins by more

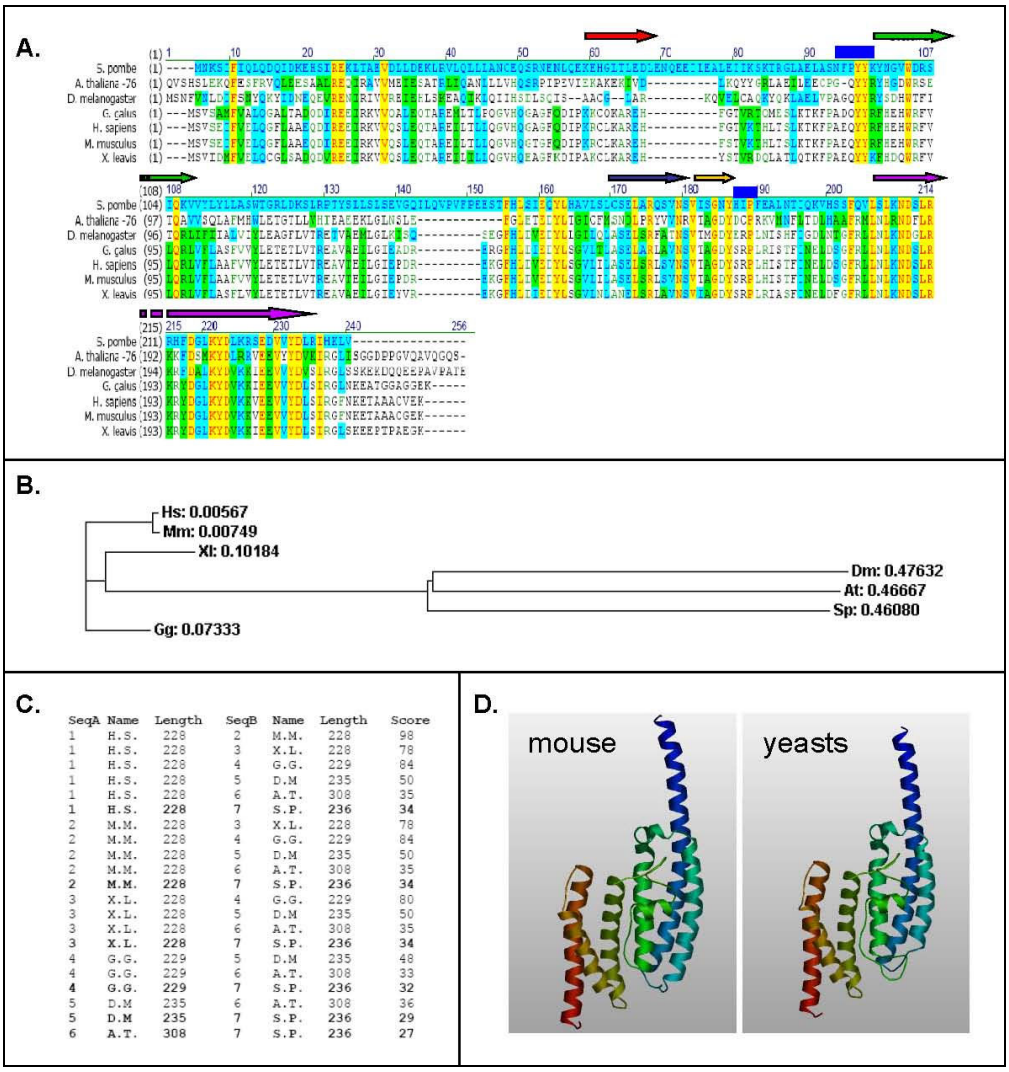

Figure 3.2. Tsn1 is evolutionary conserved in higher eukaryotes.

A. Protein sequence alignment of *S. pombe* translin with the translin sequences from higher eukaryotes generated by ClustalW2 software (www.ebi.ac.uk/ Tools/ clustalw2/). Arrows mark the recognized protein domains: red – RNA binding domain 1 (RBD1), green – RNA binding domain 2 (RBD2), blue – putative nuclear localization signal, yellow – putative GTP binding site, violet – leucin zipper domain. Blue bars mark the conserved thyrosine/proline sites. **B.** Phylogram of the translin protein sequences generated by the neighbor joining method. **C.** Table showing the pairwise alignment scores calculated between the individual translin protein sequences in A. **D.** Predicted structure of the fission yeast translin (right) modeled with SWISS-MODEL software (http://swissmodel.expasy.org/) based on the homology with mouse translin (left). *S. p.* – *Schizosaccharomyces pombe* (NP_594557), *A. t.* – *Arabidopsis thaliana* (NP_565857, the first 76 amino acids of the protein sequence are not shown in the alignment), *D. m.* – *Drosophila melanogaster* (NP_610591), *G. g.* – *Galus galus* (NP_990404), *H. s.* – *Homo sapiens* (NP_004613), *M. m.* – *Mus musculus* (NP_035780), *X. l.* – *Xhenophus laevis* (AAF65620).

than 34% (Fig. 3.2.C.). Furthermore, in the phylogram generated by the neighbor joining method the *S. pombe* translin is clustered together with the most divergent sequences from *Drosophila* and *Arabidopsis* (Fig. 3.2.B.).

Five distinct domains of Tsn1 were predicted based on the translin amino acid sequence (Laufman et al., 2005; Fig. 3.2.A.): the C-terminal terminal leucin-zipper domain (violet), the putative nuclear localization signal (dark blue), two putative RNA binding domains RBD1 (red) and RBD2 (green) and the putative GTP binding site (yellow). In general, the C-terminal part of translin is more conserved among different organisms than the N-terminal part of protein. This is in a good correlation with the evolutionarily preserved function of the C-terminal part of protein in translin oligomerization (Aoki et al., 1999; Pascal et al., 2001; Sugiura et al., 2004; Gupta et al., 2008). The more divergent N-terminal part is due to the presence of RBD domains likely involved in binding of nucleic acids (Chennathukuzhi et al., 2001; Sengupta and Rao, 2002; Jacob et al., 2004, Laufman et al., 2005). However, RBD1 and RBD2 are not well conserved between *S. pombe* and higher eukaryots (Fig. 3.2.A.), possibly due to different functional adaptation. Currently, the crystal structures of translin from *Drosophila*, mouse and humans are available (Pascal et al., 2001; Sugiura et al., 2004; Gupta et al., 2008) and can be used to predict the structure of translin homologues. Therefore, I decided to model the structure of fission yeast translin based on the homology with mouse translin (Fig. 3.2.D.) using Swiss-MODEL software (Schwede et al., 2003; Kiefer et al., 2009, Arnold et al., 2006; Kopp et al., 2004), as was already done before (Laufman et al., 2005). It is interesting to notice that the evolutionary conserved putative GTP-binding site of modeled translin is located

in the protein loop between helices 5 and 6 (Fig. 3.2.A., yellow) and that one amino acid at this site is highly conserved tyrosine (*S.p.* Y183) closely followed by a highly conserved proline (*S.p*. P186). In theory the petidyl bond proceeding the highly conserved proline *S.p*. P186 might be a putative target of Rct1 PPIase domain. The isomerisation of this particular peptidyl-prolyl bond might have a significant effect on the structure of the whole translin monomer. Importantly, at least in *Drosophila* translin mutation of this proline is detrimental for protein structure and translin oligomerization (Gupta et al., 2008). The other potential target site of Rct1 on Tsn1 is the conserved proline (*S.p.* P93) found in loop between RBD1 and RBD2, also closely followed by a highly conserved tyrosine (*S.p.* Y94). Again, the isomerization of peptidyl-prolyl bond by Rct1 at this site might have a significant impact on the protein structure and function.

3.1.3. Interaction between Rct1 and Tsn1 can be reconstituted *in vitro*

To verify interaction between Rct1 and Tsn1 observed in the yeast two hybrid assay I decided to perform *in vitro* pull down experiment with purified GST-Tsn1 protein. For this purpose I cloned *tsn1*$^{+}$ coding region into pGEX-4T-1 plasmid, expressed GST-Tsn1 fusion protein in *E. coli*, and bound the protein to glutathione sepharose beads. The protein bound to beads (Fig. 3.3.A.) was then used to pull down Rct1-HA protein from the total protein extracts of *Δrct1 + pMG1* cells overexpressing Rct1-HA protein. As shown on the Western blot with anti-HA antibodies (Fig. 3.3.B.) the purified GST-Tsn1 protein was able to pull down a small amount of Rct1-HA protein from the protein extracts, whereas the beads bound by GST only were not. This experiment clearly shows

that, at least under the experimental conditions used, the Tsn1 is able to interact with Rct1 *in vitro*.

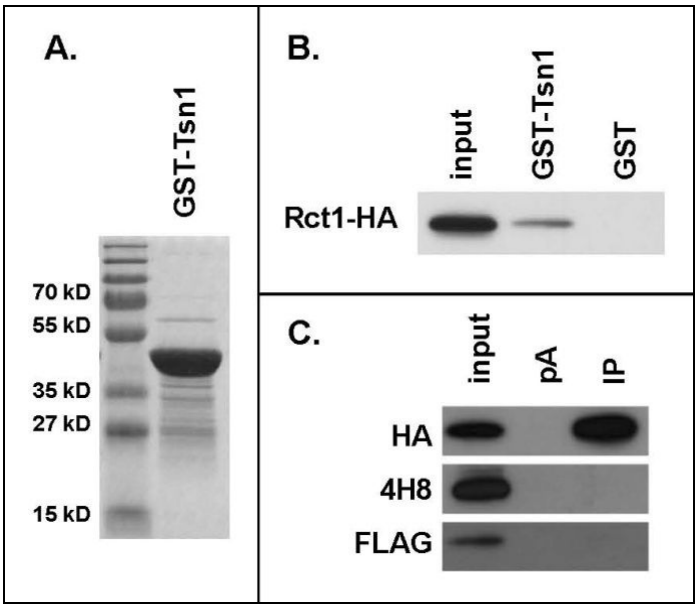

Figure 3.3. Rct1 interacts with Tsn1 *in vitro*.

A. Commasie blue stained SDS-PAGE gel of the purified GST-Tsn1 protein used in the pull down assays. **B.** Western blots with anti-HA antibodies of the pull down assay with the purified GST-Tsn1 protein and the total protein extracts from *Δrct1 + pMG1* (Rct1-HA) strain; input – total protein extract sample, GST-Tsn1 – pull down sample with the GST-Tsn1 bound to beads, GST – pull down sample with GST only bound to beads. **C.** Western blots of the co-immunoprecipitation experiment performed with the total protein extracts from *rct1-FLAG + pMGT* strain and anti-HA antibodies (HA) to immunoprecipitate Tsn1-HA. The presence of Rct1-FLAG and RNAPII in the immunoprecipitates was checked with anti-FLAG antibodies (FLAG) and anti-RNAPII CTD (4H8) antibodies (4H8): input – total protein extracts sample, pA – protein A sepharose beads only sample, IP – immunoprecipitation sample.

In the *in vitro* pull down assay described above I used recombinant GST-Tsn1 protein produced in a heterologous organism and Rct1-HA protein overexpressed in yeasts. To verify the interaction of Rct1 with Tsn1 in under the more native conditions, I decided to perform the protein co-immunoprecipitation experiment with both proteins expressed in

yeasts. For this purpose I generated rct1-FLAG strain and transformed it with pMGT plasmid. This strain expressed Rct1-FLAG protein from its native promoter on chromosome and overexpressed Tsn1-HA protein form nmt1+ promoter on pMGT plasmid. To assay for co-immunoprecipitation of the Tsn1 and Rct1 proteins from total protein extracts I immunoprecipitated Tsn1-HA with anti-HA antibodies and detected the presence of Rct1-FLAG on Western blots with anti-FLAG antibodies. Although I used the same buffer condition as in the pull down assay, I was not able to detect Rct1-FLAG protein in the complex with immunoprecipitated Tsn1-HA protein (Fig. 3.3.C.).

3.1.4. Interaction between Rct1 and Tsn1 requires all three domains of Rct1

The Rct1 is a protein with three functional domains: the N-terminal PPIase domain, the middle RRM domain and the C-terminal RS domain. To identify the parts of Rct1 protein important for the interaction with Tsn1 the individual domains of Rct1 in pMG plasmid were systematically deleted, either alone or in combinations with the other domains (Skrahina T., 2009). The resulting Rct1 PPIase only-HA, the Rct1 RRM only-HA, the Rct1 ΔRS-HA, the Rct1 ΔPPIase-HA, the Rct1 ΔRRM-HA and the Rct1 ΔPPIaseR3-HA proteins tagged with HA epitope (see Fig. 3.4. for schematic representations) were expressed in *rct1/Δrct1* genetic background and the protein extracts from these strains were used in the *in vitro* pull down assay with purified GST-Tsn1 protein (Fig. 3.5.), as already described for the full length Rct1-HA protein (Fig. 3.3.).

In these assays (Fig. 3.5.) the GST-Tsn1 protein interacted with the PPIase only-HA and the Rct1 RRM only-HA proteins, clearly showing that both the PPIase and the RRM

domain of Rct1 are able to interact with Tsn1 protein independently of each other. In accordance, the deletion of PPIase domain only (Rct1 ΔPPIase-HA) or RRM domain only (Rct1 ΔRRM-HA) did not abolish the interaction, but the deletion of PPIase domain in the combination with three point mutations in RRM domain (Rct1 ΔPPIaseR3-HA) almost diminished the interaction.

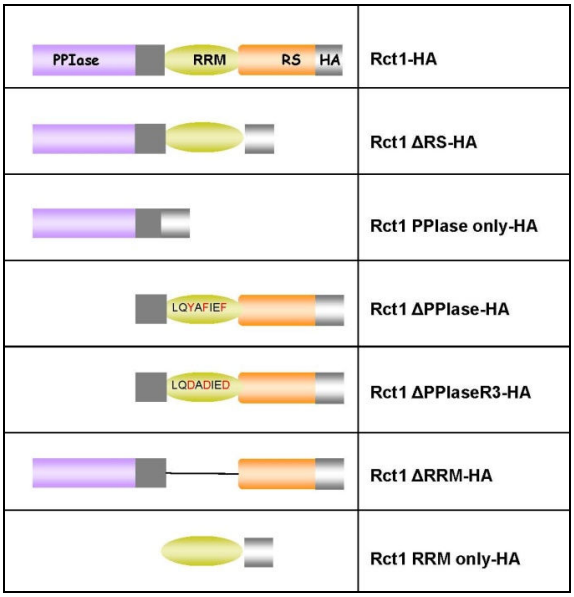

Figure 3.4. Schematic representation of Rct1 deletion mutatns (according to Skrahina T, 2009).

Surprisingly, if the RS domain of Rct1 was removed, the interaction was not detected at all, although both PPIase and RRM domains were intact, pointing at the crucial role of the RS domain in the interaction with Tsn1. Nevertheless, it is plausible that the interaction between Rct1 and Tsn1 is not mediated by the Rct1 RS domain, as there was almost no signal detected in the pull down experiment with the Rct1 ΔPPIaseR3-HA mutant protein, a protein consisting only of the RS domain and the mutated RRM domain. Therefore I concluded that the Rct1 RS domain might be important for proper

folding of Rct1 protein, and if missing, the PPIase and RRM domains are folded in a way that does not favor the interaction with Tsn1. This assumption is further supported by an observation that the interaction of Tsn1 with either PPIase or RRM domain was enhanced when they were expressed together with the RS domain. This becomes obvious if the input to pull down ratio of the individual pull down experiments is compared (see Fig. 3.5., compare input lane with GST-Tsn1 lane).

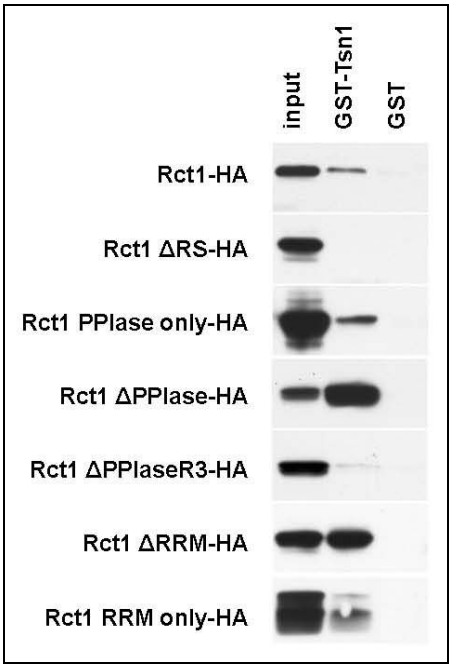

Figure 3.5. Rct1 requires all three domains for the interaction with Tsn1 *in vitro*.

Western blots with anti-HA antibodies of the pull down assays with the purified GST-Tsn1 protein and the total protein extracts from *rct1/Δrct1 + pMG1* (Rct1-HA), *rct1/Δrct1 + pMG2* (Rct1 ΔRS-HA), *rct1/Δrct1 + pMG3* (Rct1 PPIase only-HA), *rct1/Δrct1 + pMG4* (Rct1 ΔPPIase-HA), *rct1/Δrct1 + pMG4R3* (Rct1 ΔPPIaseR3 –HA), *rct1/Δrct1 + pMG5* (Rct1 ΔRRM-HA), *rct1/Δrct1 + pMG6* (Rct1 RRM only-HA) strains: input – total protein extract sample, GST-Tsn1 – pull down sample with the GST-Tsn1 bound to beads, GST – pull down sample with GST only bound to beads.

The RRM domain of a protein is usually involved in an interaction with a specific RNA target; however, in many cases it has been adapted to recognize and bind a specific protein target (Maris et al., 2005). Because both, Rct1 and Tsn1, proteins are able to bind RNA, I decided to test if the Rct1 interaction with Tsn1 detected in the pull down assay is a protein-protein interaction or an RNA mediated interaction. For this purpose I repeated the *in vitro* pull down assays as described above, but this time with the total protein extracts pre-treated with RNase A. In the experiments with the RNA free protein extracts the efficiency of GST-Tsn1 protein to pull down the Rct1 mutant proteins was exactly the same, as in the experiments with the protein extracts not pretreated with RNase A (data not shown). Therefore, I concluded that the interaction of Rct1 with Tsn1 is not mediated by RNA.

3.1.5. Rct1 and Tsn1 interact with splicing related proteins *in vitro*

Gullerova et al. (2007) showed that the deletion of *rct1*$^{+}$ gene in diploid *rct1/Δrct1* cells enhances transcription and meiosis-specific splicing of certain genes, whereas the overexpression of Rct1 from pMG1 plasmid reduces this phenotype. In addition, AtCyp59, an *Arabidopsis* homologue of Rct1, was initially identified as an interacting partner of plant SR proteins (Gullerova et al., 2006). I therefore assumed that Tsn1 could acts together with Rct1 in the regulation of splicing process. To investigate this possibility I first assayed for the interactions of Rct1 and Tsn1 with the splicing related SR proteins Srp1 and Srp2, with the core splicing factor Prp2 (an homologue of U2AF^{65}), with the main kinase of splicing factors Prp4, with the SR protein-specific kinase Dsk1 and with two evolutionary related and uncharacterized kinases Ppk5 and Ppk15.

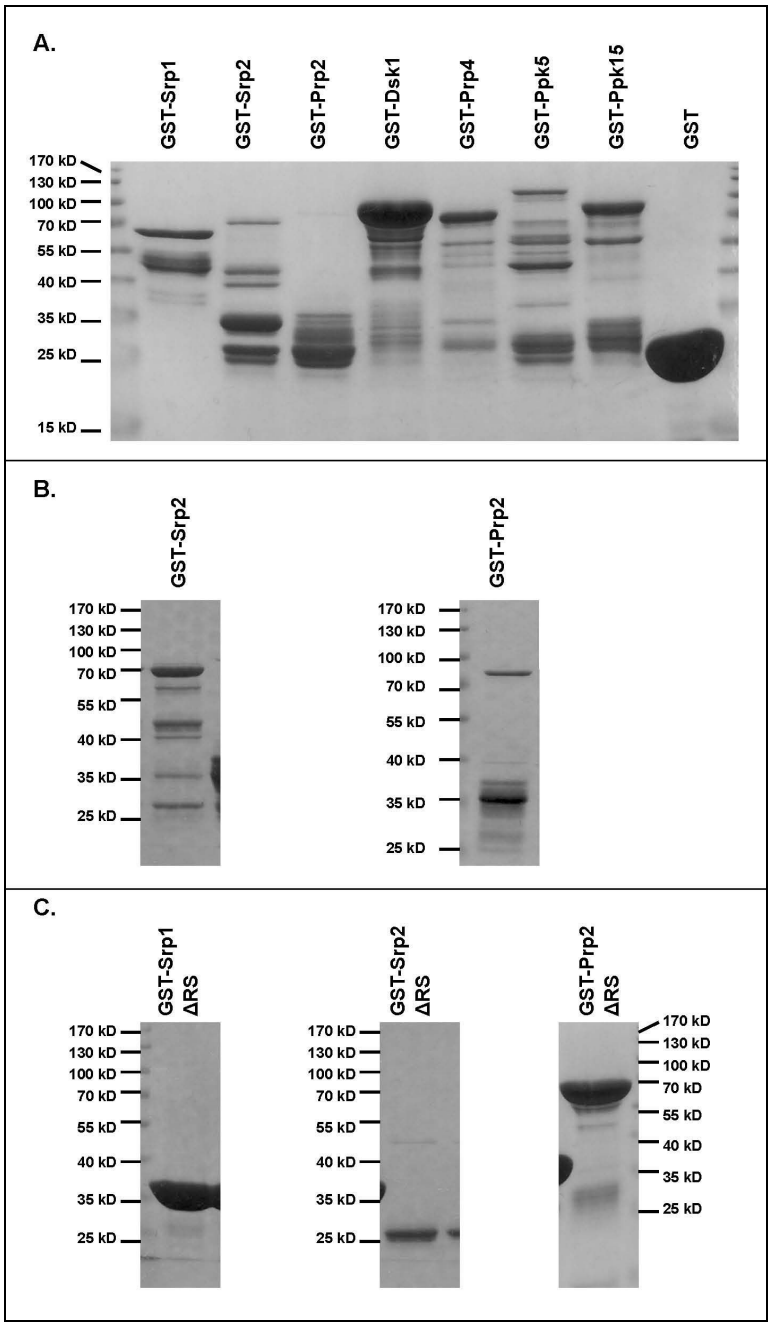

Figure 3.6. Purified GST-fusion proteins used in the pull down assays.

A. The GST-Srp1, GST-Srp2, GST-Prp2, GST-Dsk1, GST-Prp4, GST-Ppk5, GST-Ppk15 and GST proteins purified from *E. coli* BL21 strain. All of them, except of GST-Srp2 and GST-Prp2 were used for the pull down assays. **B.** The GST-Srp2 and GST-Prp2 proteins purified from *E. coli* Rosetta (DE3)pLysS strain were used in the pull down assays. **C.** The GST-Srp1 ΔRS, GST-Srp2 ΔRS and GST-Prp2 ΔRS proteins purified from *E. coli* Rosetta (DE3)pLysS strain were used in the pull down assays. See Fig. 3.7. for Western blotts of the pull down assays.

For this purpose I cloned the corresponding genes into pGEX-4T-1 plasmid, overexpressed them in *E. coli* and performed *in vitro* pull down experiments with the purified GST-tagged proteins (Fig. 3.6.) and protein extracts from wild type cells overexpressing Tsn1-HA (*wt + pMGT*) to assay for Tsn1-HA interactions or from *Δrct1 + pMG1* cells to assay for Rct1-HA interactions. Western blots with anti-HA antibodies revealed that the Rct1-HA protein interacts only with GST-Srp1, GST-Srp2, GST-Dsk1 and GST-Prp2 proteins, whereas the Tsn1-HA interacts only with GST-Prp2 and GST-Prp4 proteins (Fig. 3.7.A.). The only protein interacting with both, Rct1 and Tsn1, proteins in this assay was Prp2, an homologue of human U2AF^{65} factor. Prp2 is the large subunit of U2AF factor that is known to recognize and bind the polypyrimidine tract and 3' splice site in introns and mediate the communication of Srp2 with the spliceosome (Potashkin et al., 1993; Webb et al., 2005a). Therefore, it might be considered an excellent mediator linking Rct1 and Tsn1 with the splicing machinery. Furthermore, the interactions of Rct1 with two SR proteins, Srp1 and Srp2, support the assumption that Rct1 might be an important factor in splice site selection of certain genes. Importantly, the GST-Srp2 and GST-Prp2 were able to pull down the RNAPII from the extracts as well (Fig. 3.7.A.), suggesting that Srp2 and Prp2, as well as Rct1 (Gullerova et al., 2007) might be co-transcriptionally recruited to nascent transcript via interactions with RNAPII.

To determine the parts of Rct1 protein required for the interactions with GST-Srp1, GST-Srp2, GST-Prp2 and GST-Dsk1 proteins I performed the pull down experiments with protein extracts from *rct1/Δrct1 + pMG2*, *rct1/Δrct1 + pMG3*, *rct1/Δrct1 + pMG4*, *rct1/Δrct1 + pMG5*, *rct1/Δrct1 + pMG6* and *rct1/Δrct1 + pMG4R3* strains overexpressing

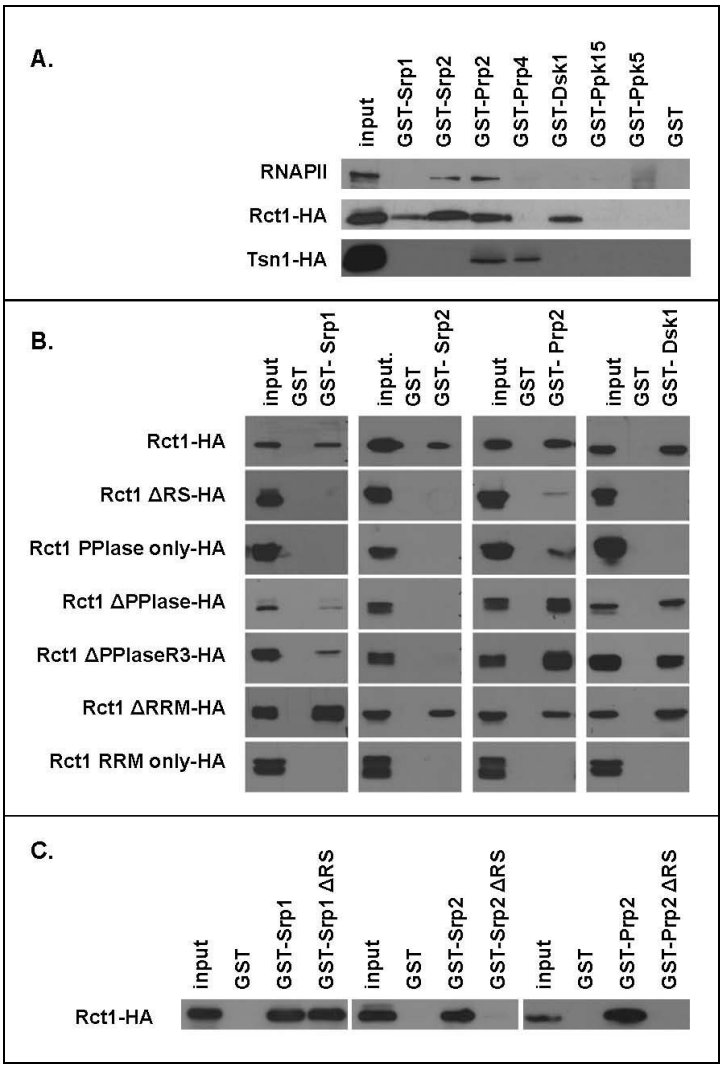

Figure 3.7. Tsn1 and Rct1 interact with splicing associated proteins.

A. Western blots with anti-HA (Rct1-HA and Tsn1-HA) and anti-RNAPII CTD (4H8) antibodies (RNAPII) of the pull down assays with the GST-Srp1, GST-Srp2, GST-Prp2, GST-Prp4, GST-Dsk1, GST-Ppk15, GST-Ppk5 proteins and the total protein extracts of *rct1/Δrct1+ pMG1* (Rct1-HA and RNAPII) and *wt + pMGT* (Tsn1-HA) strains. **B.** Western blots of the pull down assays with the GST-Srp1, GST-Srp2, GST-Prp2 and GST-Dsk1 proteins and the total protein extracts of *rct1/Δrct1+ pMG1* (Rct1-HA), *rct1/Δrct1 + pMG2* (Rct1 ΔRS-HA), *rct1/Δrct1 + pMG3* (Rct1 PPIase only-HA), *rct1/Δrct1 + pMG4* (Rct1 ΔPPIase-HA), *rct1/Δrct1 + pMG4R3* (Rct1 ΔPPIaseR3 –HA), *rct1/Δrct1 + pMG5* (Rct1 ΔRRM-HA), *rct1/Δrct1 + pMG6* (Rct1 RRM only-HA) strains to identify the Rct1 domains required for the interactions in A. **C.** Western blots with anti-HA antibodies of the pull downs with GST-Srp1, GST-Srp2, GST-Prp2, GST-Srp1 ΔRS, GST-Srp2 ΔRS, GST-Prp2 ΔRS proteins and the total protein extracts of *rct1/Δrct1+ pMG1* treated with RNase A. Input – total protein extract sample, GST-X – pull down sample with GST-X protein bound to beads, GST – pull down sample with GST only bound to beads.

different Rct1 deletion mutant proteins (see Fig. 3.4. for schemes) from *nmt1*$^{+}$ promoter. Western blots with anti-HA antibodies (Fig. 3.7.B.) show that the GST-Srp1 and the GST-Dsk1 proteins were able to pull down only those Rct1 deletion mutant proteins harboring functional RS domain, but not those lacking the RS domain (Rct1 ΔRS-HA, the Rct1 PPIase only-HA and the Rct1 RRM only-HA). Therefore, I concluded that Rct1 employs solely the RS domain for the interaction with either Srp1 or Dsk1. The interactions of GST-Srp2 and GST-Prp2 with Rct1 were more complex and required more than one domain of Rct1 (Fig. 3.7.B.). In the experiments performed, the GST-Srp2 protein interacted only with the full length Rct1-HA protein and the Rct1 ΔRRM-HA protein. It did not interact with any of the deletion mutants without the RS domain (Rct1 ΔRS-HA, Rct1 PPIase only-HA) or without the PPIase domain (Rct1 ΔPPIase-HA and Rct1 ΔPPIaseR3-HA), but also did not interact with the PPIase domain or the RRM domain alone (Rct1 PPIase only-HA, Rct1 PPIase only-HA). Based on these experiments I concluded that the PPIase and the RS domain of Rct1 must act in synergy during the interaction with Srp2. On the contrary the GST-Prp2 protein interacted with all mutant proteins tested except of the RRM only-HA protein (Fig. 3.7.B.). This means that Rct1 employs both, the PPIase and the RS domain, but not the RRM domain, for the interaction with the GST-Prp2. However, the PPIase and the RS domain are able to interact independently of each other.

To determine if the RS domains of Srp1, Srp2 and Prp2 proteins are required for the interactions with Rct1 I created new plasmids expressing the truncated GST-tagged proteins: GST-Srp1 ΔRS, GST-Srp2 ΔRS and GST-Prp2 ΔRS (see Fig. 3.8. for schemes) and I used them in the pull down assays with Δrct1 + pMG1 protein extracts treated with

RNase A, as described before. Western blots with anti-HA antibodies revealed that whereas GST-Srp1 ΔRS was able to interact with Rct1, the GST-Srp2 ΔRS and GST-Prp2 ΔRS was not (Fig. 3.7.C.). Therefore, I concluded that the interaction between Srp1 and Rct1 is mediated by Srp1 RRM domain and Rct1 RS domain, whereas Rct1 interacts with Srp2 RS and Prp2 RS domains employing both PPIase and RS domains.

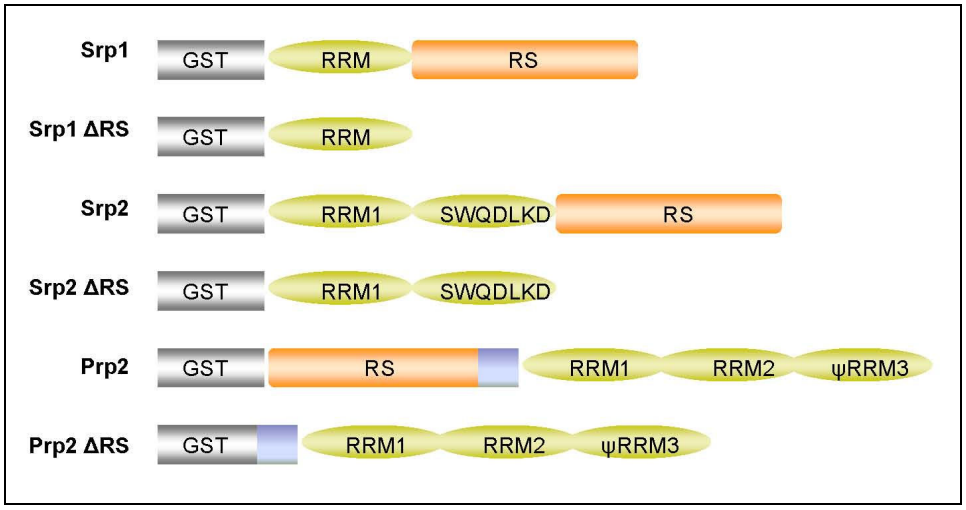

Figure 3.8. Schemes of Srp1, Srp2 and Prp2 proteins used for the pull down assays.

Schematic representation of domain organization of full length and ΔRS Srp1, Srp2 and Prp2 proteins that were used in the pull down assays in Fig. 3.7.C.. GST – position of GST tag, RRM – typical RRM motif with two RNP motifs, ψRRM – pseudo RRM of Prp2, SWQDLKD – an atypical RRM of Srp2 without the RNPs, but with the SWQDLKD motif, RS – domain rich in arginine-serine dipeptides, blue - a short linker region of Prp2 .

3.1.6. Srp1 and Srp2 are highly phosphorylated nuclear proteins

The Srp1 and Srp2 are the only characterized SR proteins in the fission yeasts. The larger Srp2 has two N-terminal RRM domains and one C-terminal RS domain (Lutzelberger et al., 1999). The Srp1 is smaller and is composed of one N-terminal RRM domain and one C-

terminal RS domain (Gross et al., 1998; Tang et al., 1998). Both, Srp1 and Srp2, are targets of Dsk1 protein kinase that phosphoryltes their RS domains (Tang et al., 1998, 2000, 2002, 2007). The phosphorylation of Srp1 RS and Srp2 RS domains by Dsk1 seems to regulate the entry of the proteins into nucleus, as well as their interaction with each other (Tang et al., 2007). The available information about Srp1 and Srp2 proteins are based mainly on *in vitro* experiments and overexpression studies. To further characterize Srp1 and Srp2 proteins in living cells I generated strains with FLAG-tagged *srp1*$^{+}$ and *srp2*$^{+}$ genes on the chromosome expressing *srp1-FLAG* and *srp2-FLAG* proteins, respectively (see Fig. 3.9.A. for schematic representation of tagged alleles). The *srp2*, but not *srp1*, is an essential gene (Lutzelberger et al., 1999). Therefore, I checked whether the addition of FLAG tag affects the growth rates of the resulting strains. However, I did not observe any difference in growth rates of wild type, s*rp1-FLAG* and s*rp2-FLAG* strains neither in YES nor in EMM medium at 32°C (Fig. 3.9.B.), indicating that these strains could be used for physiological and biochemical experiments.

Next, I compared the expression of Srp1-FLAG and Srp2-FLAG proteins. As expected, when the total protein extracts of s*rp1-FLAG* and s*rp2-FLAG* strains were probed on Western blots with the anti-FLAG antibodies, there was always only one discreet band recognized. The molecular mass of Srp1-FLAG specific band was approximately 47 kDa and the molecular mass of Srp2-FLAG was approximately 55 kDa. Then I compared the expression levels of Srp1-FLAG, Srp2-FLAG and Rct1-FLAG proteins in the exponentially

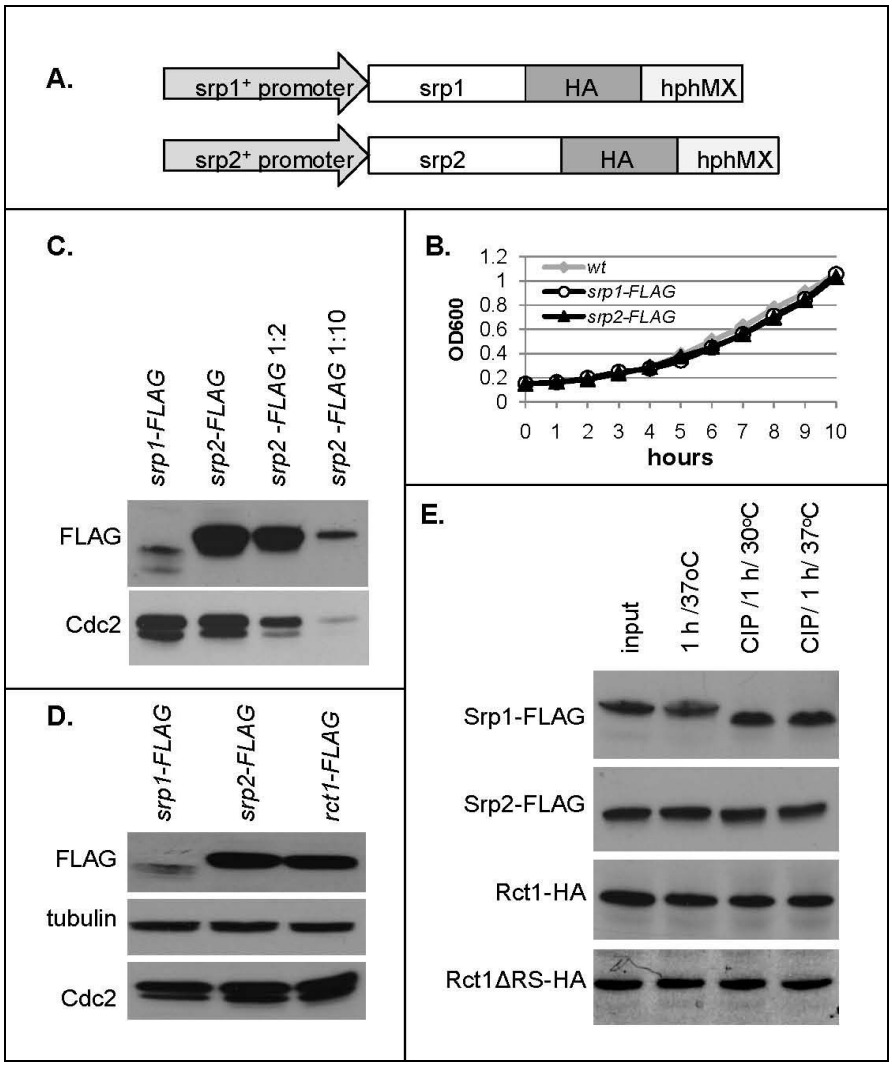

Figure 3.9. Characterization of Srp1-FLAG and Srp2-FLAG proteins.

A. Schematic representation of *srp1* and *srp2* genomic loci of the *srp1-FLAG* and *srp2-FLAG* strains. **B.** Growth curves comparing the growth rates of *srp1-FLAG* and *srp2-FLAG* strains in YES medium at 30°C. **C.** Western blots with anti-FLAG and anti-Cdc2 (loading control) antibodies comparing the expression of Srp1-FLAG and Srp2-FLAG proteins in *srp1-FLAG* and *srp2-FLAG* strains. Loaded are either not diluted (*srp1-FLAG, srp2-FLAG*), 1:2 diluted (*srp2-FLAG* 1:2) and 1:10 diluted (*srp2-FLAG* 1:10) total protein extracts from strains *srp1-FLAG* and *srp2-FLAG*. **D.** Western blots with anti-FLAG, anti-tubulin (loading control) and anti-Cdc2 (loading control) antibodies comparing the expression of Srp1-FLAG, Srp2-FLAG and Rct1-FLAG in *srp1-FLAG*, *srp2-FLAG* and *rct1-FLAG* strains, respectively. Loaded are the total protein extracts of the same protein concentration. **E.** Western blots of the dephosphorylation assay with anti-FLAG (for Srp1-FLAG and Srp2-FLAG) and anti-HA (for Rct1-HA and Rct1 ΔRS-HA) antibodies. Loaded are the total protein extract of *srp1-FLAG*, *srp2-FLAG*, *rct1-HA* and *rct1 ΔRS-HA* strains (input) incubated either with 1U/µl CIP at 30°C and 37°C or without the CIP at 37°C for 1 hour.

growing s*rp1-FLAG,* s*rp2-FLAG* and *rct1-FLAG* strains in the YES medium at 30°C. I observed that the expression of Srp2-FLAG is approximately ten times higher than the expression of Srp1-FLAG protein as revealed by serial dilutions of Srp2-FLAG extracts (Fig. 3.9.C.), whereas the expression of Rct1-FLAG is roughly the same as the expression of Srp2-FLAG (Fig. 3.9.D.).

The actual molecular masses of Srp1-FLAG and Srp2-FLAG were slightly higher than predicted by SGD (*Schizosaccharomyces pombe* GeneDB database at http://old.genedb.org/genedb/pombe/), possibly due to an extensive phosphorylation of their RS domains. However, Srp1-FLAG and Srp2-FLAG proteins appear as discrete bands on the Western blots arguing that the whole population of Srp1 or Srp2 in the cell is evenly phosphorylated (Fig. 3.9.C., D. and E.; in C. the lover band in Srp1-FLAG sample is likely a protein degradation product). To determine whether Srp1-FLAG and Srp2-FLAG proteins exist in the cell predominantly in a phosphorylated or a nonphosphorylated form I treated the total protein extracts of *srp1-FLAG* and *srp2-FLAG* strains with the calf intestine phosphatase (CIP, 1 U/ µl) at 30°C or 37°C for 1 hour and compared the protein migration of CIP treated, CIP untreated and input samples on 10% SDS-PAGE gel. As obvious from the Western blot with anti-FLAG antibodies (Fig. 3.9.E.), the treatment of protein extracts with CIP resulted in significant Srp1-FLAG specific band shift, arguing that the Srp1 protein is predominantly highly phosphorylated in the cells. On the contrary, the Srp2-FLAG specific band was only modestly shifted in the CIP treated protein extracts, although the same conditions for separation were used. This

experiment shows that although the Srp2 protein is phosphorylated in the cells, it is less phosphorylated than the Srp1 protein.

The RS domain of Rct1 might be, in principle, also phosphorylated by the Dsk1 kinase, or by another RS domain targeting kinase. To test the assumption that the RS domain of Rct1 is phosphorylated *in vivo*, I treated the *rct1-HA* and *rct1 ΔRS-HA* protein extracts with the CIP, as described above, and compared the protein migration of treated and untreated samples. However, I could not observe any Rct1-HA specific band shift on Western blots with anti-HA antibodies in CIP treated samples, when compared to untreated samples (Fig. 3.9.E.). Therefore I concluded that the RS domain of Rct1 is not highly phosphorylated *in vivo*, but rather exists in a nonphosphorylated or a low phosphorylated form.

The actual phosphorylation status of the Srp1 RS and Srp2 RS domains is thought to regulate the localization of Srp1 and Srp2 proteins in the cell (Tang et al., 2007). According to this study, if Dsk1 kinase is active in the cells the overexpressed Srp2 protein is found predominantly in the nucleus, whereas the overexpressed Srp1 protein is mostly localized in the cytoplasm. This suggests that the Dsk1-dependent phosphorylation is crucial for the localization of the Srp1 and Srp2 proteins; however, these localization studies were done under overexpression conditions. To reveal the localization of Srp1-FLAG and Srp2-FLAG proteins under the endogenous expression levels I immunostained the formaldehyde fixed cells with the anti-FLAG antibodies and ALEXA 568 coupled secondary antibodies and compared the distribution of ALEXA 568-

specific signal with the HOECHST-specific DNA signal. In both *srp1-FLAG* and *srp2-FLAG* strains I observed co-localization of Srp1 or Srp2 proteins with the nuclear DNA (Fig. 3.10.A.). I did not observe any ALEXA 568-specific signal outside of the nucleus, neither in the s*rp1-FLAG* nor in the s*rp2-FLAG* cells. This observations suggest that Srp1 and Srp2 are nuclear proteins.

To further investigate the localization of Srp1 and Srp2 *in vivo* I decided to prepare strains expressing fluorescently labeled Srp1 and Srp2 proteins. For this purpose the *tdTomato::hphMX* cassette from pFA6a-hphMX6 plasmid was inserted into the chromosome at the same location downstream of $srp1^{+}$ and $srp2^{+}$ as the *FLAG::hphMX* cassette before. By this approach I obtained *srp1-tdTomato* strain, but no *srp2-tdTomato* strain. I also failed to tag the $srp2^{+}$ with the GFP tag, suggesting that the large protein tags, such as tdTomato or GFP, could interfere with the function of otherwise essential Srp2 protein. This is well consistent with the data from global ORFeome analysis that also failed to obtain strain expressing YFP labeled Srp2 protein (Matsuyama et al., 2006). The *srp1-tdTomato* strain expressed the Srp1-tdTomato protein of expected size of app. 130 kDa, detected on the Western blot with anti-dsRed antibodies (Fig. 3.10.C.). Importantly, the entire fluorescent signal of Srp1-tdTomato protein in living cells localized to the nucleus (Fig. 3.10.B.), further supporting my observation that Srp1 is entirely a nuclear protein.

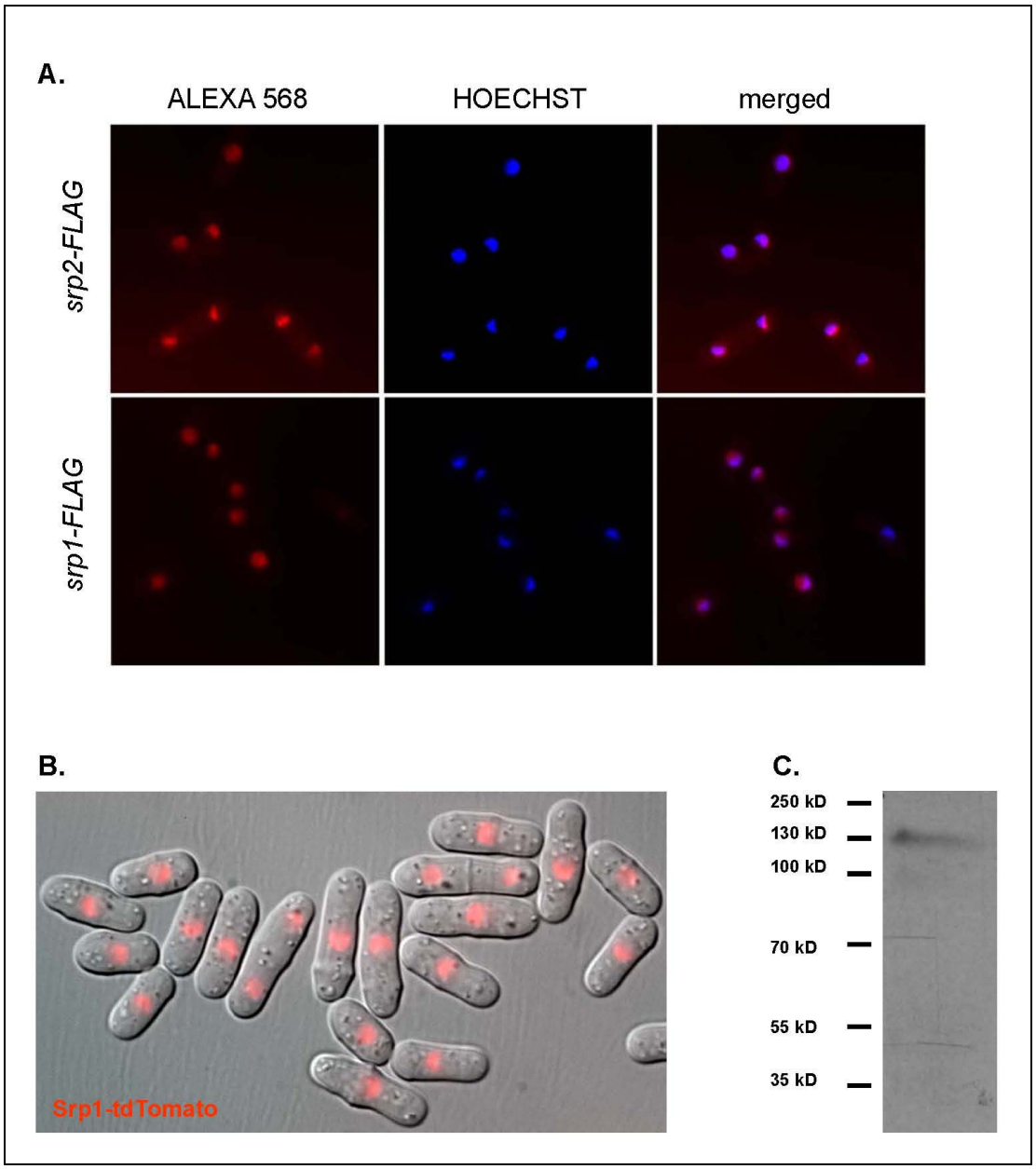

Figure 3.10. Srp1 and Srp2 are pure nuclear proteins

A. Immunolocalization of Srp1-FLAG and Srp2-FLAG proteins in formaldehyde fixed *srp1-FLAG* and *srp2-FLAG* cells probed with anti-FLAG antibodies and Alexa 568 conjugated secondary antibodies (left), DNA localization after staining with Hoechst (middle) and the merged pictures (right). **B.** Fluorescent microscopy picture of s*rp1-tdTomato* live cells expressing the Srp1-tdTomato fluorescent protein from the native s*rp1*$^{+}$ promoter merged with DIC picture of the same cells. **C.** Western blot with anti-dsRed antibodies and s*rp1-tdTomato* total protein extracts.

I also attempted to verify the *in vitro* interactions detected between the Rct1 and GST-Srp1 and GST-Srp2 proteins (Fig. 3.7.) by co-immunoprecipitation of the proteins from the yeast protein extracts. For this purpose I prepared *rct1-HA srp1-FLAG* and *rct1-HA srp2-FLAG* strains and used them for co-immunoprecipitation experiments with anti-FLAG (Fig. 3.11.A.) or anti-HA (Fig. 3.11.B.) antibodies. However, I was neither able to co-immunoprecipitate the proteins with each other, nor with the RNAPII (Fig. 3.11.).

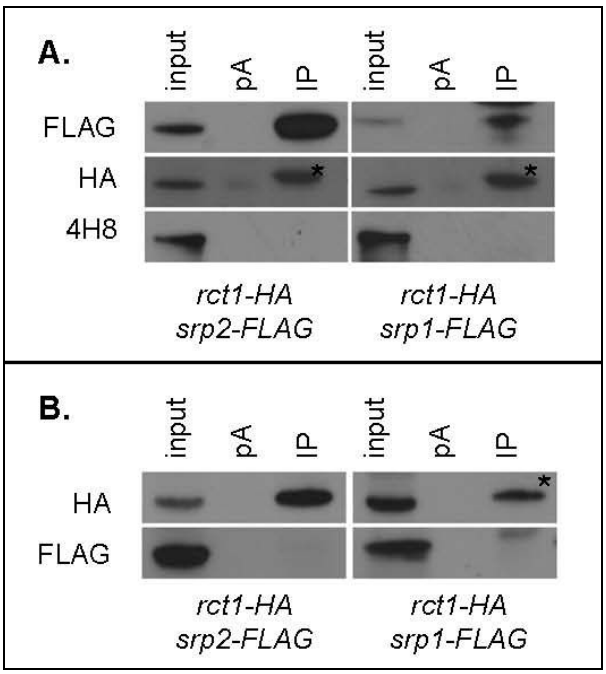

Figure 3.11. Srp1-FLAG and Srp2-FLAG do not co-immunoprecipitate with Rct1

A. Immunoprecipitation of the Srp2-FLAG and Srp1-FLAG with anti-FLAG antibodies from the total protein extracts of *srp2-FLAG rct1-HA* and *srp1-FLAG rct1-HA* strains. **B.** Immunoprecipitation of Rct1-HA with anti-HA antibodies from the total protein extracts from *srp2-FLAG rct1-HA* and *srp1-FLAG rct1-HA* strains. FLAG – Western blots with anti-FLAG antibodies to detect Srp2-FLAG or Srp1-FLAG, HA – Western blots with anti-HA antibodies to detect Rct1-HA, 4H8 – Western blots with anti-RNAPII CTD (4H8) antibodies to detect RNAPII, input – total protein sample, pA – extracts incubated with protein A sepharose beads, IP – immunoprecipitation sample, asterisks marks the immunoglobulin bands.

3.2. ROLE OF Rct1 RS DOMAIN IN REGULATION OF TRANSCRIPTION AND SPLICING

3.2.1. The C-terminal RS domain of Rct1 protein is important for its function

The common part of Rct1 protein involved in the protein-protein interactions described above is the C-terminal RS domain. This domain is important for the interactions with Srp1, Srp2, Prp2 and Dsk1 and also essential part of Rct1 in the interaction with Tsn1 (Figs. 3.5. and 3.7.B.). Furthermore the deletion of RS domain in the GAL4-AD-Rct1 ΔRS construct used in the yeast two hybrid assay activated the transcription of reporter genes in the presence of GAL4-BD domain alone, while the full length Rct1 protein did not (Fig. 3.1.C.). This all together point at a possibly important role of the RS domain for the function of the whole Rct1 protein.

To investigate the role of RS domain *in vivo* I prepared two strains. In the first I replaced *rct1*$^{+}$ gene with *rct1::HA::kanMX* sequence from pMG1 plasmid and in the other with *rct1 ΔRS::HA::kanMX* sequence from pMG2 plasmid. By this approach I obtained *rct1-HA* and *rct1 ΔRS-HA* strains expressing Rct1-HA and Rct1 ΔRS-HA proteins under the control of native *rct1*$^{+}$ promotor (see Fig. 3.12.A. for schemes of resulting loci). First, I compared the growth rates of generated strains in YES and EMM media at 30°C. Graphs in (Fig. 3.12.B.) show that both strains have the same growth rates as wild type in both types of media, and hence I assumed that the RS domain of Rct1 is a nonessential part of otherwise essential protein. Knock down of Rct1 expression results in the branched multicellular structures (Gullerova et al., 2007). Therefore, I have also compared cell morphology of

the exponentially growing strains, but I did not notice any difference between *rct1-HA* and *rct1 ΔRS-HA* strains, neither in the cell shape nor in the septation (Fig. 3.12.C.).

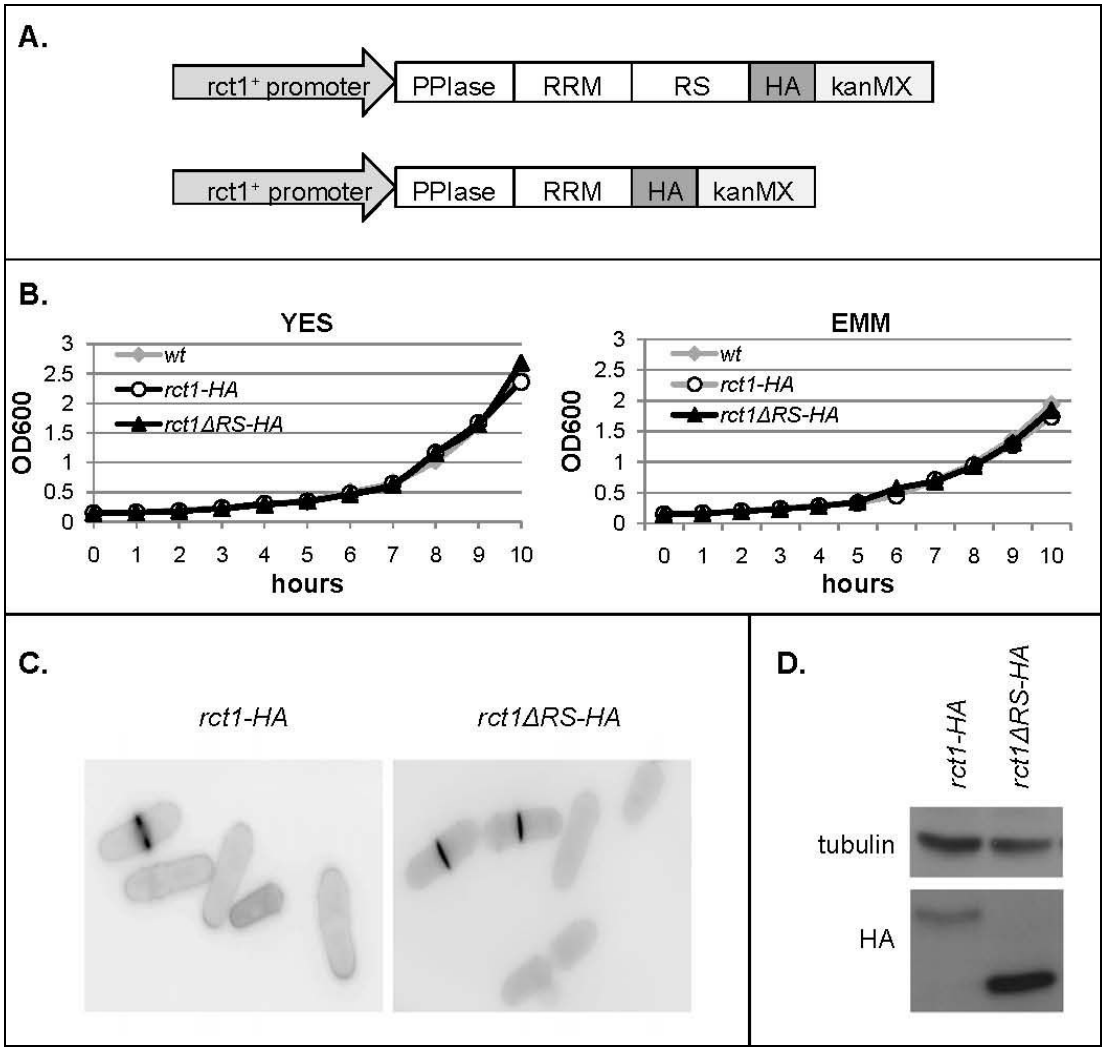

Figure 3.12. Characterization of *rct1-HA* and *rct1 ΔRS-HA* strains

A. Schematic representation of the *rct1* genomic loci in the *rct1-HA* and *rct1 ΔRS-HA* strains. **B.** Comparison of the growth rates of *wt, rct1-HA* and *rct1 ΔRS-HA* strains in YES (left) or EMM (right) medium at 30°C. **C.** Fluorescent microscopy pictures of the aniline blue stained *rct1-HA* and *rct1 ΔRS-HA* cells grown in YES medium at 30°C. **D.** Western blots with anti-HA and anti-tubulin (loading control) antibodies comparing the expression levels of Rct1-HA and Rct1 ΔRS-HA proteins in *rct1-HA* and *rct1 ΔRS-HA* strains grown in YES medium at 30°C.

I also compared the Rct1 protein expression levels in the total protein extracts of the new strains on the Western blots with anti-HA antibodies. I observed that the Rct1 ΔRS-HA protein expression is reproducibly enhanced when compared to Rct1-HA protein expression, although the expression of tubulin control was not affected (Fig. 3.12.D.). This suggested that the Rct1 RS domain is important either for Rct1 protein stability or for the Rct1 mRNA expression.

From the experiments with the strains overexpressing Rct1-HA and Rct1 ΔRS-HA from pMG1 and pMG2 plasmids is evident that the RS domain is important for nuclear localization of Rct1 protein (Lorkovic ZJ, unpublished results). To investigate the effect of RS domain deletion on the Rct1 protein localization in the cells expressing Rct1 from its native promoter I immunostained formaldehyde fixed *rct1-HA* and *rct1 ΔRS-HA* cells with anti-HA antibodies and ALEXA 568 coupled secondary antibodies. Unfortunately, the fluorescence signal obtained was repeatedly too low to be detected with fluorescent microscope (not shown). However, I obtained strong and reliable nuclear signal when the *rct1-FLAG* strain was stained with anti-FLAG antibodies and ALEXA 568 coupled secondary antibodies (Fig. 3.13.). Therefore, I suppose that the low signal intensity of *rct1-HA* and *rct1 ΔRS-HA* samplas was rather a result of inefficient anti-HA antibody staining of the samples then a result of the low level of Rct1 and Rct1 ΔRS protein expression.

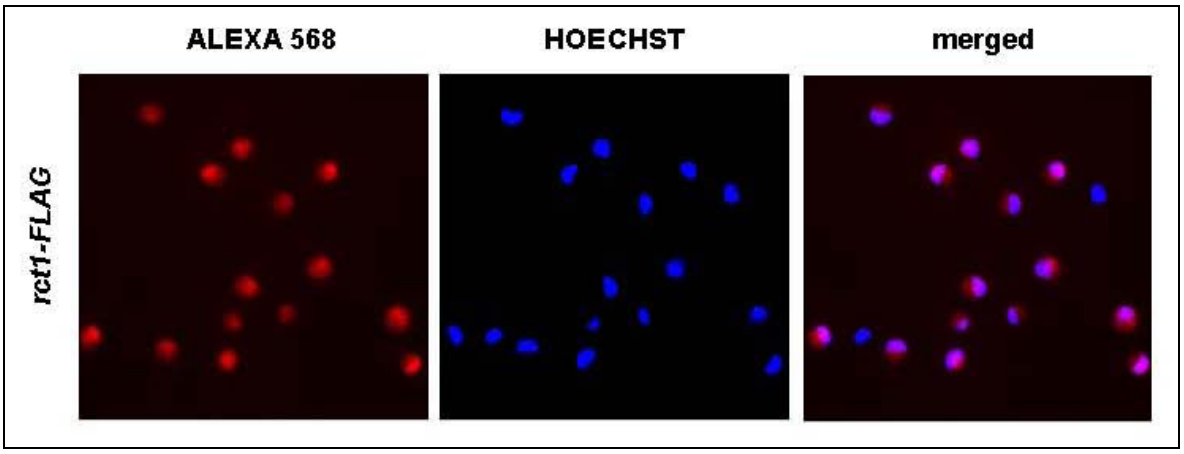

Figure. 3.13. Rct1-FLAG protein localizes to nucleus

Fluorescent microscopy pictures of formaldehyde fixed *rct1-FLAG* cells stained with Hoechst dye for DNA localization and immunostained with anti-FLAG antibodies and ALEXA 568 coupled secondary antibodies for Rct1-FLAG localization.

3.2.2. RNAPII distribution along intron containing gene *dis2* is affected by Rct1 RS domain deletion

Rct1 was characterized as an important factor of RNAPII mediated transcription that regulates RNAPII phosphorylation in dosage-dependent manner (Gullerova et al., 2007). The PPIase domain of Rct1 interacts with the CTD domain of RNAPII (Gullerova et al., 2007) and with the CTD Ser2-specific kinases Cdk9 and Lsk1. Furthermore it has been shown that Rct1 regulates Cdk9 activity *in vitro* on CTD substrate (Skrahina T., 2009). Here I showed that Rct1 can interact with several splicing factors *in vitro* by its C-terminal RS domain (Fig. 3.7.). Therefore, I hypothesized that the RS domain of Rct1 might be required to couple the RNAPII transcription with the splicing of introns. In my working

model the Rct1 could act as a mediator transmitting signals from the transcription machinery to the splicing machinery, or vice versa from the splicing factors to the transcription machinery.

To test this hypothesis I designed chromatin immunoprecipitation experiments (ChIPs) to investigate a possibility that the RNAPII distribution along the intron containing genes is affected by the Rct1 RS domain deletion. For this purpose I selected three intron containing genes: the low expressed *dis2* and *osm1* genes and highly expressed *rho1* gene and designed PCR probes along their coding regions (see Fig. 3.14. for schematic representations of the loci). The genes were selected based on two criteria: a single large intron at the 5' end of mRNA and no transcriptionally active chromatin in proximity of gene's coding region. The probes were designed along the whole transcriptional units; however, the special care was taken to design probes in introns and regions just upstream and downstream of introns to acquire the information on splicing related dynamics of analyzed factors.

To get an overall picture of the transcriptional dynamic along the selected genes I first assayed for RNAPII distribution. For this purpose I first fixed *rct1-HA* and *rct1 ΔRS-HA* cells with formaldehyde, then immunopreciptiated the RNAPII with anti-RNAPII CTD (4H8) antibodies and finally quantified the DNA bound in complex with immunoprecipitated RNAPII by SYBR Green-based real-time PCRs. For the graphical representations of results the obtained relative DNA values were normalized to the relative DNA values at an unrelated genomic region encoding 22S rRNA (marked as C in

Fig. 3.14.). The distribution of RNAPII signal along the *dis2* and *osm1* transcriptional units in *rct1-HA* cells was similar (Fig. 3.14. compare graphs in A and B): the RNAPII signal was low at the promoter (D1, O1 in Fig. 3.14.A. and B.), increased at the beginning of ORF (D2, O2 in Fig. 3.14.A. and B.), high around the intronic sequence (D3, D4, O3 in Fig. 3.14.A. and B.), dropping down downstream of intron (D5, O4 in Fig. 3.14.A. and B.) and again increasing towards the end of gene (D6, D7, O6 in Fig. 3.14.A. and B.), suggesting that the two genes are transcribed in a similar way. The RNAPII distribution was slightly different along the highly transcribed *rho1* gene. Here, the RNAPII signal did not decrease downstream of the intron (R4 in Fig. 3.14.C.), but remained high, as is typical for highly transcribed genes (Wilhelm et al., 2009). In addition the RNAPII signal along the *osm1* and *rho1* genes was exactly the same in *rct1 ΔRS-HA* as in *rct1-HA* cells (Fig. 3.14. compare blue and purple bars). By contrast, I noticed that the RNAPII signal at the position D5 located 300-400 bp downstream of *dis2* intron, was reproducibly almost two times higher in *rct1 ΔRS-HA* cells than in *rct1-HA* cells (Fig. 3.14.A.). The increased RNAPII signal was not observed at the positions D4 and D6 which are in the close proximity of D5, as well as at any other position along *dis2* gene. I speculated that the difference in RNAPII signal at the position D5 differently reflects the regulated activity of RNAPII in this region. It is possible that the RNAPII is here fully active only when the full length Rct1 is expressed and is stalled when the Rct1 RS domain is deleted.

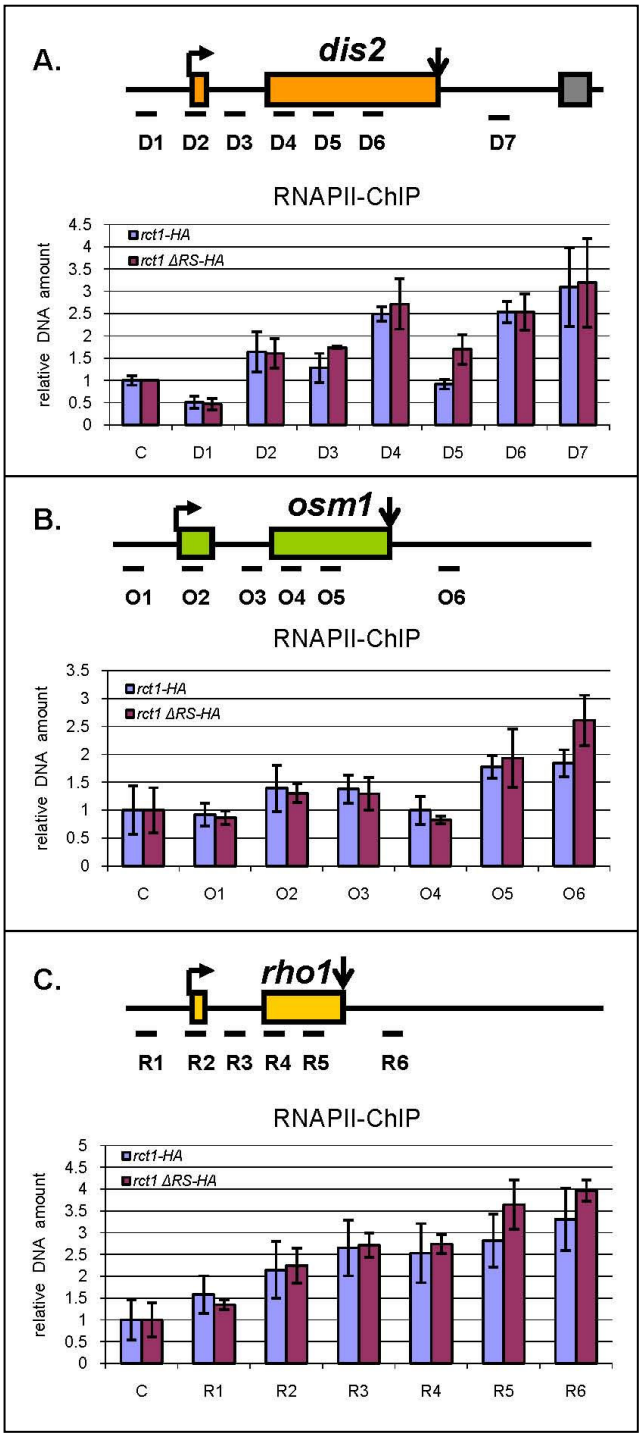

Figure 3.14. RNAPII occupancy downstream of intron in *dis2* gene is altered upon the Rct1 RS domain deletion

Results of RNAPII-ChIP experiments showing the RNAPII occupancy along the *dis2*, *rho1* and *osm1* intron containing genes in *rct1-HA* and *rct1 ΔRS-HA* strains. Shown are the relative values of the DNA amount precipitated with anti-RNAPII CTD (4H8) antibodies. All relative DNA values were normalized to the signal obtained at an unrelated rDNA region (C). **A.** Schematic representation of *dis2* transcriptional unit (orange). The positions of DNA fragments amplified by qPCRs are indicated by dashes below (D1-D7). Graphical representation of the RNAPII-ChIP results showing the occupancy of RNAPII along the *dis2* transcriptional unit. Arrows mark the beginning and the end of gene's ORF. The results shown are based on five independent biological repeats. **B.** Schematic representation of *osm1* transcriptional unit (green). The positions of DNA fragments amplified by qPCRs are indicated by dashes below (O1-O6). Arrows mark the beginning and the end of gene's ORF. Graphical representation of RNAPII-ChIP results showing the occupancy of RNAPII along the *osm1* transcriptional unit. The results shown are based on three independent biological repeats. **C.** Schematic representation of *rho1* transcriptional unit (yellow). The positions of DNA fragments amplified by qPCRs are indicated by dashes below (R1-R7). Arrows mark the beginning and the end of gene's ORF. Graphical representation of RNAPII-ChIP results showing the occupancy of RNAPII along the *rho1* transcriptional unit. The results shown are based on three independent biological repeats.

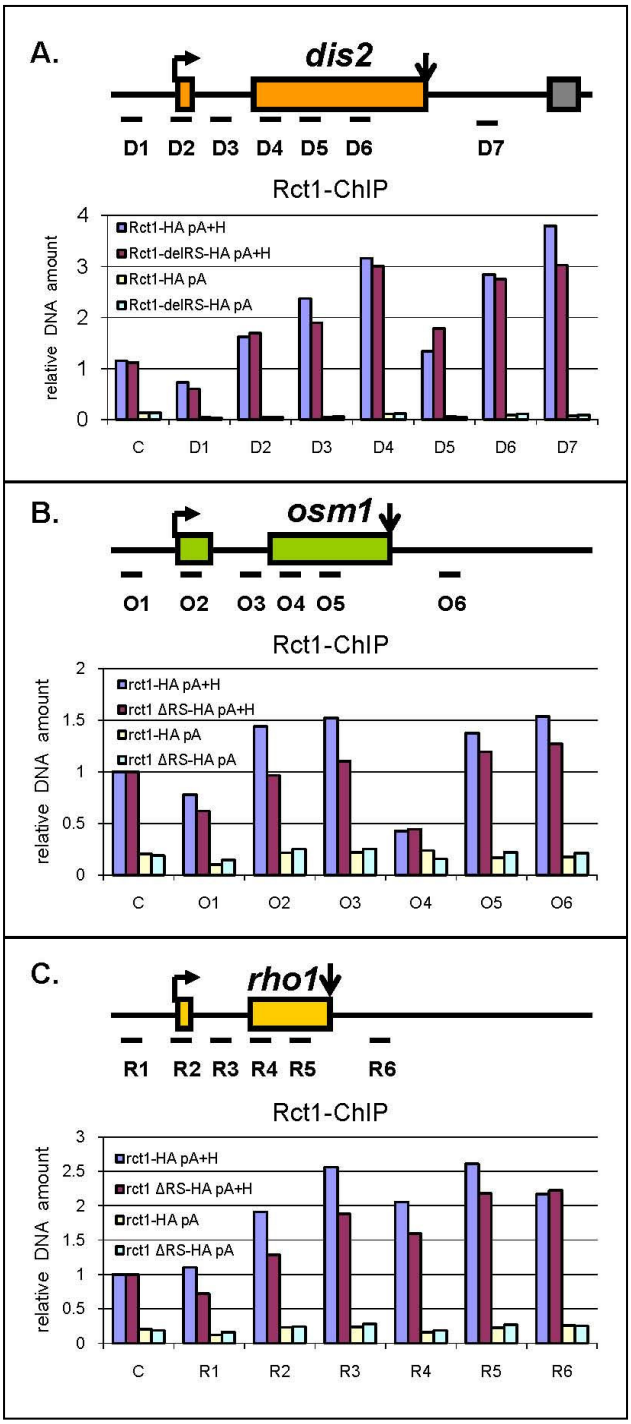

Figure 3.15. Distribution of Rct1-HA protein along *dis2, rho1* and *osm1* gene is not changed upon the Rct1 RS domain deletion

Results of Rct1-ChIP experiment showing the occupancy of Rct1-HA and Rct1 ΔRS-HA along the *dis2* (**A**), *osm1* (**B**) and *rho1* (**C**) intron containing genes in *rct1-HA* and *rct1 ΔRS-HA* strains. Shown are the relative values of the DNA amount precipitated with anti-HA (12CA5) antibodies (pA+H). For the comparison the relative DNA values obtained in a parallel experiment without the antibody (pA) are shown. All relative DNA values were normalized to signal obtained at an unrelated rDNA region (C). Results of one of three independent biological experiments are shown. The schematic representations are as in Fig. 3.14..

It is possible that the deletion of Rct1 RS domain in *rct1 ΔRS-HA* strain interferes with the association of Rct1 with the transcriptionally active chromatin. To investigate whether the distribution of Rct1-HA along *dis2*, *rho1* and *osm1* genes is affected by the Rct1 RS domain deletion I performed ChIP experiments with both strains as before, but with anti-12CA5 antibodies to precipitate Rct1-HA and Rct1 ΔRS-HA proteins. Although I repeated the experiment three times I did not observe any reproducible difference between the distribution of Rct1-HA and Rct1 ΔRS-HA proteins along any of the genes tested (Fig. 3.15. compare blue and purple bars). The little differences between Rct1-HA and Rct1ΔRS-HA that can be observed in Fig. 3.15.B. and C. were not confirmed in the other two repetitions of the experiment. Interestingly, the distribution profile of Rct1-HA and Rct1 ΔRS-HA proteins along these genes closely resembled that of RNAPII (compare Fig. 3.14. with Fig. 3.15.), suggesting that both, Rct1 and Rct1 ΔRS, proteins ''travel'' along these genes in a complex with RNAPII.

3.2.3. Splicing efficiency is not affected by Rct1 RS domain deletion

The increased amount of RNAPII at the position D5 at *dis2* gene in *rct1 ΔRS-HA* strain might be a result of inefficient coupling of splicing reaction to RNAPII transcription. To test whether the splicing reaction is affected by the Rct1 RS domain deletion I designed RT-PCR experiments to compare the splicing efficiency levels of *dis2*, *osm1* and *rho1* mRNAs in *rct1-HA* and *rct1 ΔRS-HA* strains.

First, I quantified the amount of spliced and unspliced mRNAs in the reverse transcribed RNA samples by the semiquantitative PCR reactions (see Fig. 3.16.A for schematic

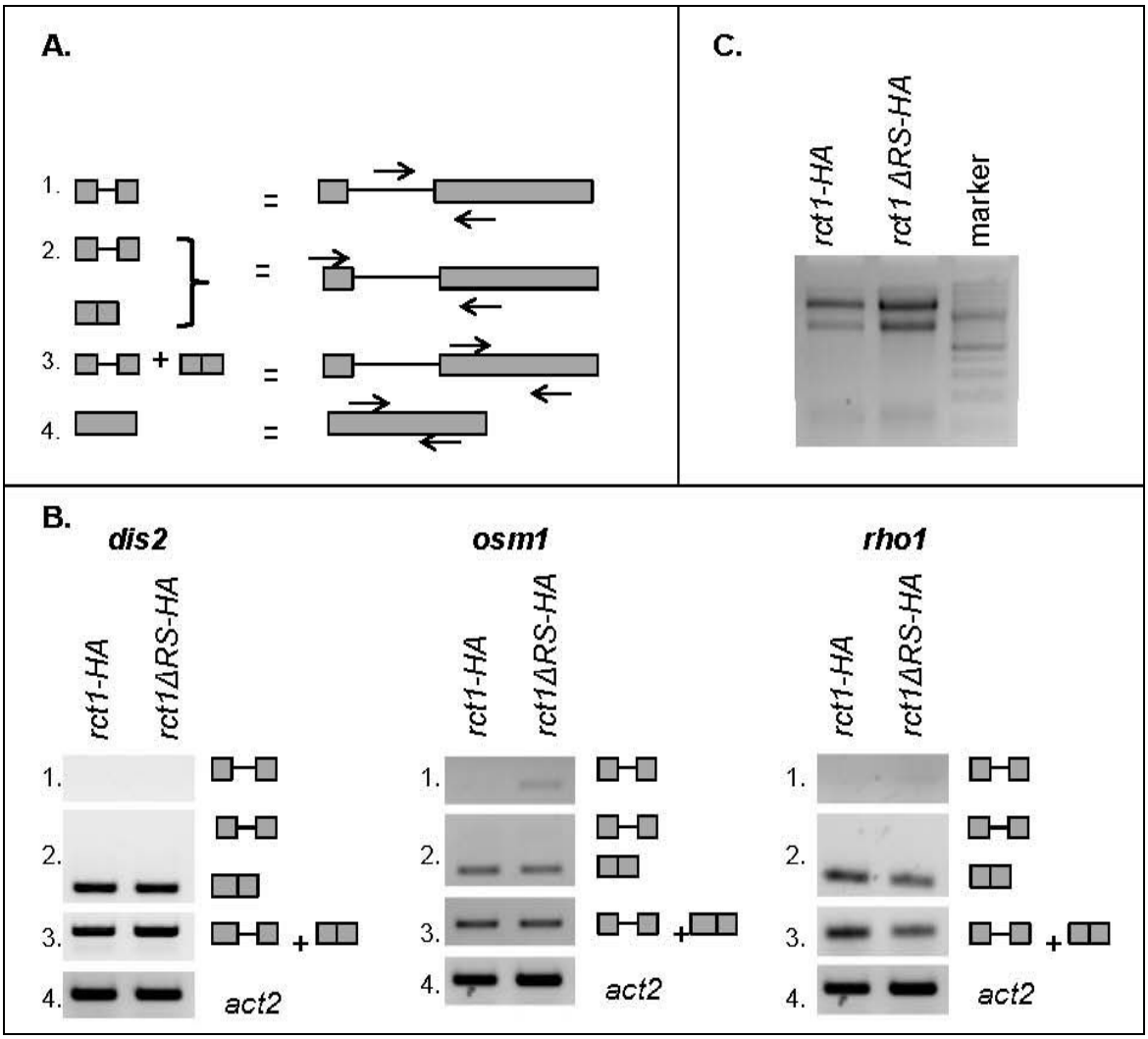

Figure 3.16. Splicing of *dis2, rho1* and *osm1* mRNA is not affected by Rct1 RS domain deletion.

A. Scheme of the PCR primer pairs (1 - 4) used for the semiquantitative amplification of spliced and unspliced mRNAs of *dis2*, *osm1* and *rho1* genes (1-3) and control act1 gene (4) after the reverse transcription. **B.** Ethidium bromide stained agarose gels showing the PCR products obtained after the amplification of *dis2*, *osm1, rho1* and *act1* cDNAs with the primer pairs 1 - 4 from A. **C.** Ethidium bromide stained agarose gel showing the separated total RNA of *rct1-HA* and *rct1 ΔRS-HA* strains. The visible bands correspond to the abundant ribosomal rRNA of *S. pombe*.

representations of primer probes). As can be seen in Fig. 3.16.B., the signal obtained after amplification of the region corresponding to the exon 2 of either *dis2*, *osm1* or *rho1* mRNAs was the same in both *rct1-HA* and *rct1 ΔRS-HA* strains (Fig. 3.16.B. , panel 3). This shows that the deletion of Rct1 RS domain does not affect the steady-state level of these mRNAs in the cell. I obtained similar results whit primer pairs priming upstream and downstream of *dis2*, *osm1* or *rho1* introns, i.e. primers pairs amplifying both spliced and unspliced mRNA variants (Fig. 3.16.B., panel 2). The signal corresponding to spliced mRNA variants was the same in *rct1-HA* and *rct1 ΔRS-HA* strain in all three analyzed genes (Fig. 3.16.B., bottom of panel 2). However, no detectable signal corresponding to unspliced mRNA was obtained (Fig. 3.16.B., top of panel 2). When the primer pairs priming within the intronic sequences were used, only a low intensity signal corresponding to unspliced mRNAs of *osm1* and *rho1* genes, but not *dis2* gene, was obtained (Fig. 3.16.B., panel 1). Amplification of a segment of intronless actin mRNA resulted in the same signal intensity in both strains (Fig. 3.16.B., panel 4), showing that the comparable amounts of total RNA were analyzed.

Next, I quantified the amount of total mRNA and unspliced mRNA of *dis2*, *osm1* and *rho1* genes in the same RT-PCR samples, but this time by the quantitative real-time PCRs. For this purpose I used the same primer pairs as for ChIP experiments (i.e. D4, R5, O5, D2, R3 and O3). The probes were selected to amplify either the exonic regions (D4, R5, O5) or the intronic regions (D2, R3, O3) of *dis2*, *osm1* and *rho1* mRNAs (see Fig. 3.17.A for schematic representation of primer probes). The graphs in Fig. 3.17. show that the amount of total (Fig. 3.17.B.), as well as unspliced (Fig. 3.17.C.), mRNA of *dis2*, *osm1* and

rho1 genes did not change upon the deletion of Rct1 RS domain. The data also show that, as expected, the expression level of *dis2* and *osm1* genes were lower than the expression level of *rho1* gene (Fig. 3.17.B. compare D4 and O5 with R5). Together the results show that the deletion of Rct1 RS domain does not affect the pre-mRNA splicing of the analysed genes.

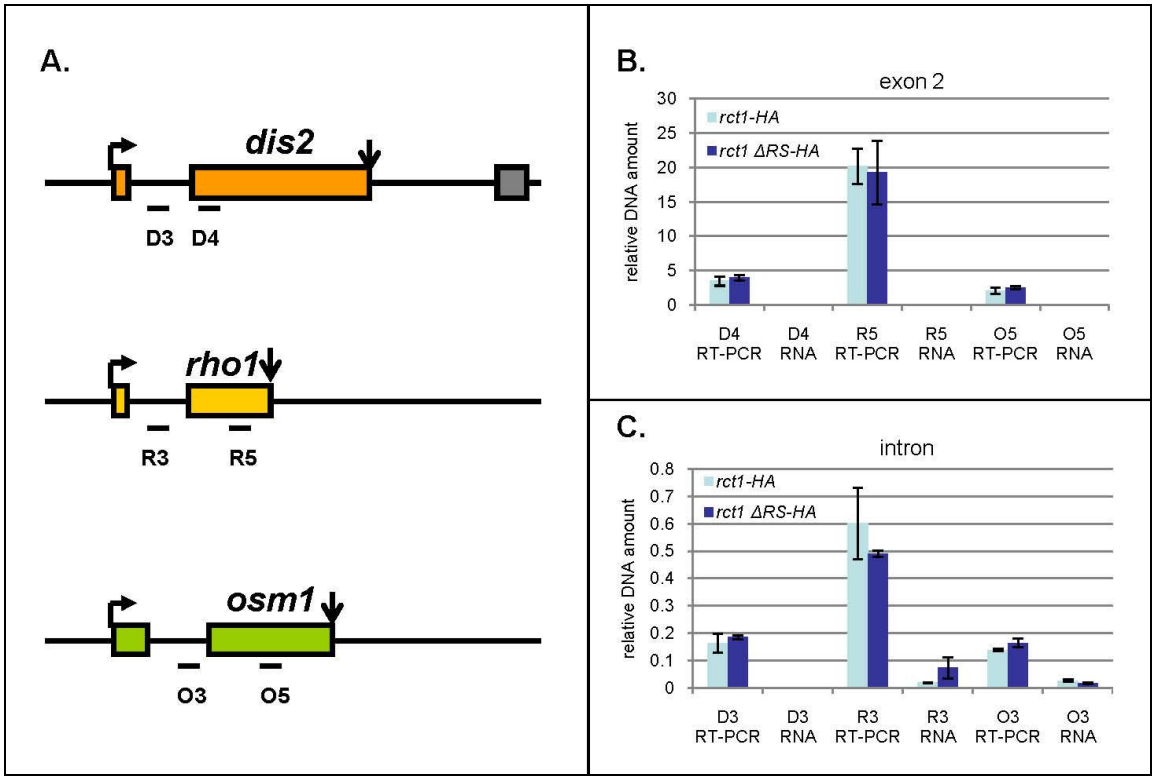

Figure 3.17. Splicing efficiency of *dis2, rho1* and *osm1* mRNA is not affected by Rct1 RS domain deletion as revealed by real time PCRs analysis.

A. Schematic representations of *dis2, osm1* and *rho1* transcriptional units and positions of primer probes used for real-time PCR amplification. **B., C.** Graphs showing the relative amounts of total (exon 2) (B.) and unspliced (intron) (C.) *dis2*, *rho1* and *osm1* mRNAs detected by real-time PCR of the reverse transcribed mRNA (RT-PCR) and total, not reverse transcribed RNA (RNA) using the probes D4, R5, O5, D2, R3, O3 as depicted in A. Error bars represent standard deviations of three independent biological repeats.

Interestingly, the amount of total RNA isolated from *rct1 ΔRS-HA* strain was repeatedly 2-3 times higher than the amount of RNA isolated from the same amount of cells of *rct1-HA* strain, as became evident after separation of isolated RNA on 2% agarose gel followed by visualization of rRNA with ethidium bromide (Fig. 3.16.C.). The difference was further supported by spectrophotometric measurements (not shown). This is important, because in accordance with RT-PCR protocol the experiments were performed with the same amount of total RNA and therefore corrected for the original difference in total RNA amounts between the two strains. Yet, there was no difference in *dis2*, *osm1* and *rho1* mRNA levels between the *rct1-HA* and *rct1 ΔRS-HA* strains in RT-PCR experiments shown in Figs. 3.16.B. and 3.17.B.. Although, the difference in total RNA amounts between the two strains seems to be significant, it likely does not result from the altered RNAPII transcription, because it affects the levels of rRNA proportionally to that of mRNA.

3.2.4. Deletion of RS domain does not affect the distribution of Srp2 along *dis2*, *osm1* and *rho1* transcriptional units

The Rct1 associated with the transcriptionally active RNAPII might associate with the splicing factors Srp1 and Srp2 in two ways: it can either facilitate their recruitment to the nascent transcript or it can modulate their activity once they are already associated with RNAPII and with the nascent transcript. To test the first possibility I prepared four strains expressing Rct1-HA and Rct1 ΔRS-HA proteins in the combination with Srp1-FLAG or Srp2-FLAG proteins, i.e. *rct1-HA srp1-FLAG*, *rct1 ΔRS -HA srp1-FLAG*, *rct1-HA srp2-FLAG* and *rct1 ΔRS-HA srp2-FLAG* strains. I used these strains to map the distribution of Srp1-

FLAG and Srp2-FLAG along *dis2* gene in ChIP experiment with anti-FLAG antibodies. If my hypothesis was correct than the distribution of Srp1 or Srp2 would be altered by the Rct1 RS domain deletion. First, my results showed that only Srp2-FLAG protein, but not Srp1-FLAG protein, is enriched along *dis2* transcriptional unit, if compared to the background values (Fig. 3.18. compare A with B and pA with pA+F). Second, as is evident from the graph in Fig. 3.18.B., there is no significant difference in the Srp2-FLAG distribution along *dis2* gene in the *rct1 ΔRS-HA srp2 FLAG* cells when compared with the *rct1-HA srp2-FLAG* cells, except of position D5. However, this difference was not obvious in four of five independent experiment repetitions and therefore it cannot be considered significant. In *rct1-HA srp2-FLAG* cells the Srp2-ChIP signal was almost at the background level on the promoter of *dis2* gene (D1 in Fig. 3.18.B.), two times higher at the beginning of gene (exon 1) and in the middle of intron (D2, D3 in Fig. 3.18.B.), five times higher at the intron – exon 2 junction (D4 in Fig. 3.18.B.), dropping down downstream of intron (two times higher than at the promoter, D5 in Fig. 3.18.B.) and increasing further downstream at the exon 2 (D6 in Fig. 3.18.B.) and in the 3' untranslated region (D7 in Fig. 3.18.B.). This distribution of Srp2-FLAG protein closely resembled that of the RNAPII, suggesting that the RNAPII and Srp2 proteins are in a complex when associated with the *dis2* gene (compare Fig. 3.14.A. with Fig. 3.18.B.). Furthermore, I have also compared the distribution of Srp2-FLAG along *osm1* and *rho1* genes, but did not find any significant difference in the Srp2 distribution between the *rct1-HA srp1-FLAG* and *rct1 ΔRS-HA srp1-FLAG* strains (Fig. 3.18. compare blue and yellow bars in C. and D.), suggesting that the Rct1 RS domain is not needed to recruit the Srp2 protein to the transcribing RNAPII.

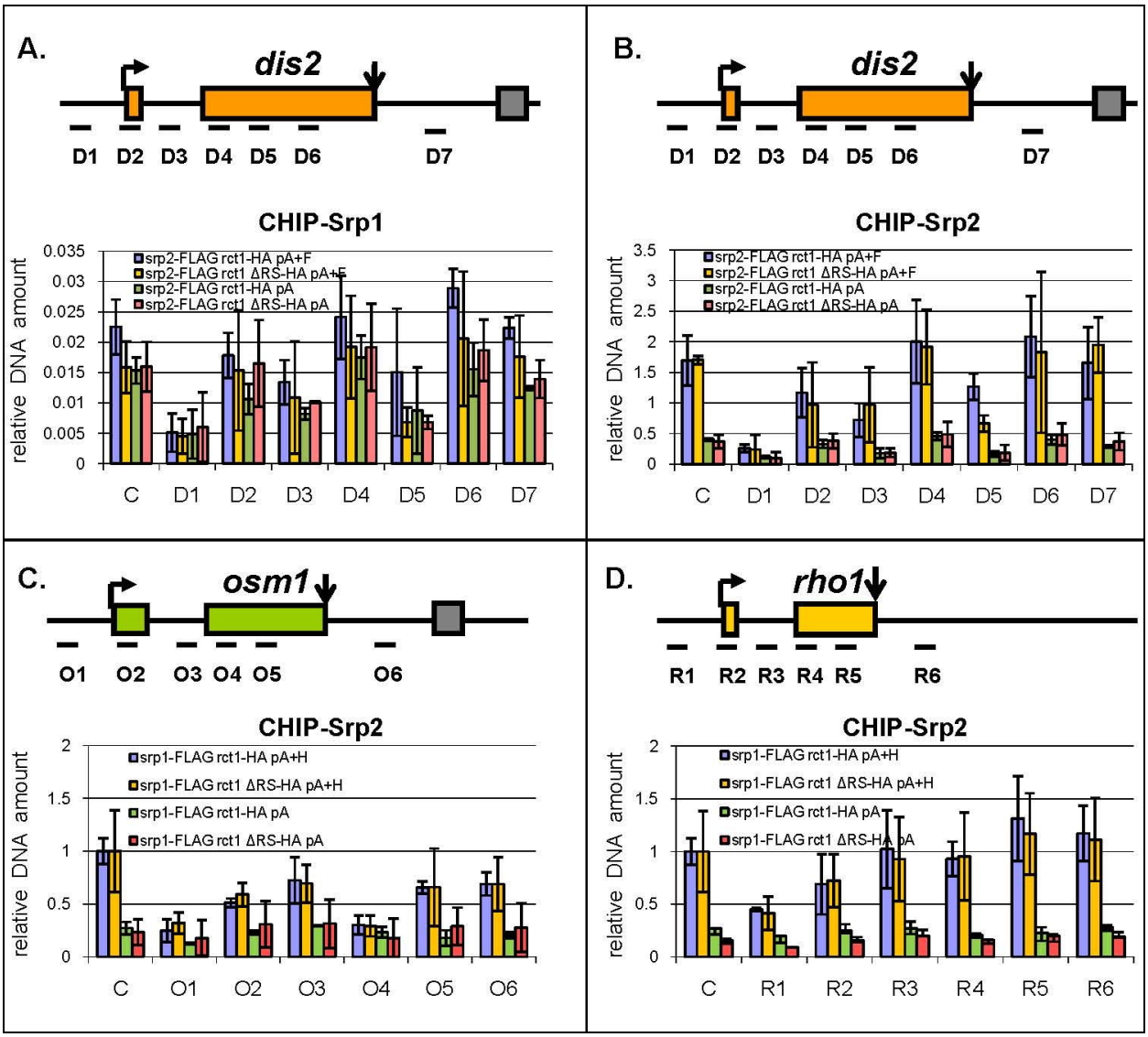

Figure 3.18. Srp2, but not Srp1, is associated with transcriptionally active chromatin

A. Results of the Srp1-ChIP experiment showing the occupancy of Srp1-FLAG along *dis2* transcriptional unit in *srp1-FLAG rct1-HA* and *srp1-FLAG rct1 ΔRS-HA* strains. The results shown are based on two independent biological experiments. **B, C, D.** Results of the Srp2-ChIP experiment showing the occupancy of Srp2-FLAG along *dis2* (B)*, osm1* (C) and *rho1* (D*)* transcriptional units in *srp2-FLAG rct1-HA* and *srp2-FLAG rct1 ΔRS-HA* strains. Results are based on five (B) or two (C and D) independent biological experiments. Shown are the relative values of DNA amounts precipitated with anti-FLAG antibodies (pA+F). For comparison the relative DNA values obtained in the parallel experiment without the antibody (pA) are shown. All relative DNA values were normalized to the signal obtained at an unrelated rDNA region (C). Schematic representations are as in Fig. 3.14..

3.2.5. RNAPII distribution along intron containing gene *dis2* is also affected by the overexpression of Rct1 protein

As already stated, the deletion of Rct1 RS domain resulted in the increased expression of truncated Rct1 protein when compared to the expression level of full length protein (Fig. 3.12.D.). Therefore, I cannot exclude a possibility that the increased RNAPII signal at the position D5 at *dis2* gene in RNAPII-ChIP experiments is a result of the overexpression of truncated protein, which consists of the PPIase and RRM domains. To test this possibility I performed similar RNAPII-ChIP experiment along the *dis2* gene as already described above, but this time with cells overexpressiong the Rct1-HA protein (i.e. *Δrct1 + pMG1* cells grown in EMM without thiamine) or with the same cells after the Rct1-HA depletion (i.e. grown in EMM with thiamine) and compared them with the wild type cells and *Δrct1* cells expressing Rct1 ΔPPIase-HA protein from $nmt1^{+}$ promotor (i.e. *Δrct1 + pMG4* cells grown in EMM without thiamine). The overexpression of Rct1-HA protein, similar to the deletion of RS domain in *rct1 ΔRS-HA* cells (Fig. 3.14.A.), resulted in the accumulation of RNAPII signal at the position D5 (Fig. 3.19.A), whereas the distribution of RNAPII along the rest of *dis2* gene was not significantly affected (Fig. 3.19.A.). By contrast, there was no accumulation at the D5 possition, when the Rct1-HA was depleted by the addition of thiamine, or when the Rct1 ΔPPIase-HA protein was expressed instead of the full length protein (Fig. 3.19.A.). It is important to mention that the overall amount of RNAPII precipitated DNA corresponding to *dis2* transcriptional unit was not increased upon the Rct1-HA overexpression.

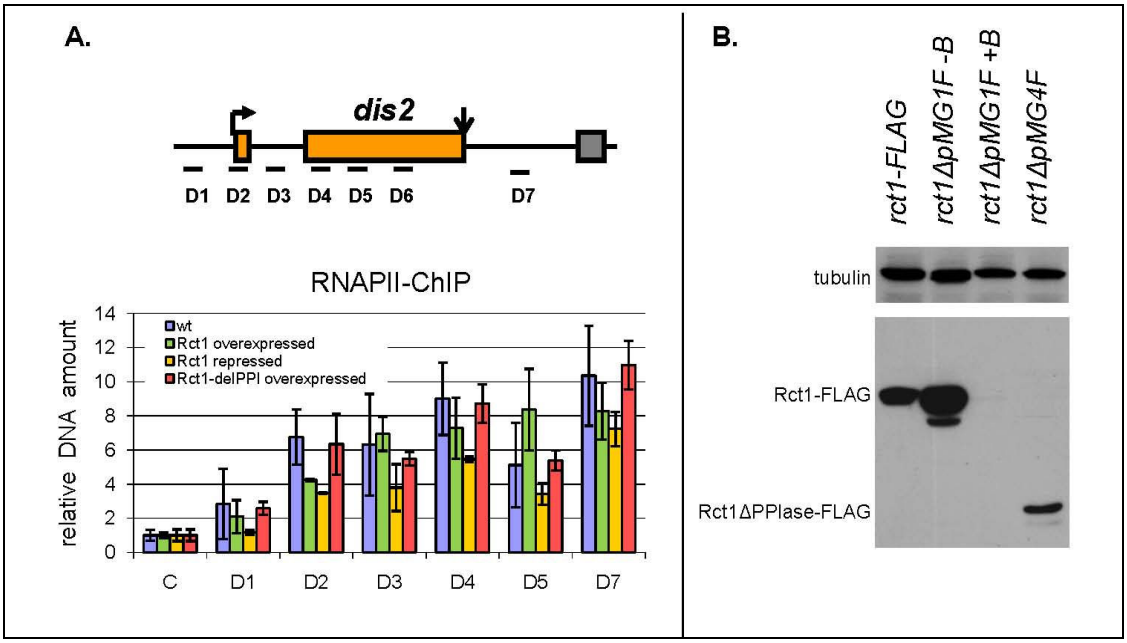

Figure 3.19. RNAPII occupancy at the position D5 at *dis2* gene is also altered upon the Rct1-HA overexpression

A. Results of the RNAPII-ChIP experiment showing the RNAPII occupancy along *dis2* gene in *wt* cells, in *Δrct1+ pMG1* cells overexpressing Rct1-HA in the EMM medium, in *Δrct1+ pMG1* cells after repression of the overexpression of Rct1-HA by the addition of thiamine and in *Δrct1+ pMG4* cells overexpressiong Rct1 ΔPPIase-HA protein in the EMM medium. Shown are the relative values of DNA amounts precipitated with anti-RNAPII CTD (4H8) antibodies. All relative DNA values were normalized to the signal obtained at an unrelated rDNA region (C). The results shown are based on two independent biological experiments. The schematic representation of *dis2* gene is as in Fig. 3.14.A.. **B.** Western blot with anti-tubulin and anti-FLAG antibodies and protein extracts from *rct1-FLAG*, *Δrct1 + pMG1F* (overexpressing Rct1-FLAG protein) and *Δrct1 + pMG4F* (expressing Rct1 ΔPPIase-FLAG protein) cells grown in the presence (+B) or absence (-B) of thiamine.

All together this experiment shows that the overexpression of Rct1-HA has similar effect on the RNAPII distribution along the *dis2* gene as the deletion of Rct1 RS domain. The depletion of Rct1-HA in the cells, as well as the overexpression of Rct1 ΔPPIase-HA peptide resulted in the wild type RNAPII distribution along *dis2* gene, suggesting that it is the overexpression of PPIase domain that is necessary for RNAPII accumulation at the

position D5. However, from similar experiment we know that the level of Rct1 ΔPPIase-FLAG protein under the overexpression conditions (when *nmt1*$^{+}$ promotor is on) is significantly lower than the endogenous Rct1 protein level under the same conditions (Fig. 3.19.B. compare *rct1-FLAG* and *rct1ΔpMG4F*). Therefore I cannot exclude the possibility that the RNAPII accumulation at the position D5 is a result of the RRM domain overexpression.

3.3. PHENOTYPIC ANALYSIS OF TRANSLIN DELETION AND OVEREXPRESSION STRAINS

3.3.1. Proper expression of Tsn1 protein is not important for viability of haploid cells

The *tsn1* is referred to be a nonessential gene and its deletion does not seem to have any effect on the cell viability (Jaendling et al., 2008) or, as referred by the others (Laufman et al., 2005), it only slightly enhances the growth rate of *Δtsn1* spores. For my experiments I generated two *Δtsn1* deletion strains. To obtain the *Δtsn1(kan)* strain I first replaced one allele of *tsn1*$^{+}$ gene in the wild type diploid cells with the *kanMX* cassette amplified from pMG plasmid and then prepared the haploid strains by tetrad dissection of the sporulated diploid cells. To obtain the *Δtsn1(nat)* strain I directly replaced the ORF of *tsn1*$^{+}$ gene with the *natMX* cassette from pAG25 plasmid in the wild type haploid cells.

First, I compared the cell morphology of exponentially growing *Δtsn1(kan)* and *Δtsn1(nat)* strains with the haploid wild type strain in both EMM and YES media at 30°C, but I did not observe any differences (Fig. 3.20. A. and B.). I also did not observe any difference in the growth rates of created strains and wild type strain (Fig. 3.20. C and E.). Next, I examined the effect of Tsn1-HA overexpression on the growth rates of the haploid wild type and *Δtsn1(nat)* cells. For this purpose I transformed the cells with empty pMG plasmid and pMGT plasmid overexpressing Tsn1-HA protein, and compared their growth rates with the growth rates of the wild type and *Δtsn1(nat)* strains in EMM medium at 30°C (i.e. under the conditions when *nmt1*$^{+}$ promoter is on). However, I did

not observe any difference in the growth rates (Fig. 3.20.D.), nor in the cell morphology (Fig. 3.20.A. and E.), when compared to *wt* or *Δtsn1(nat)* cells.

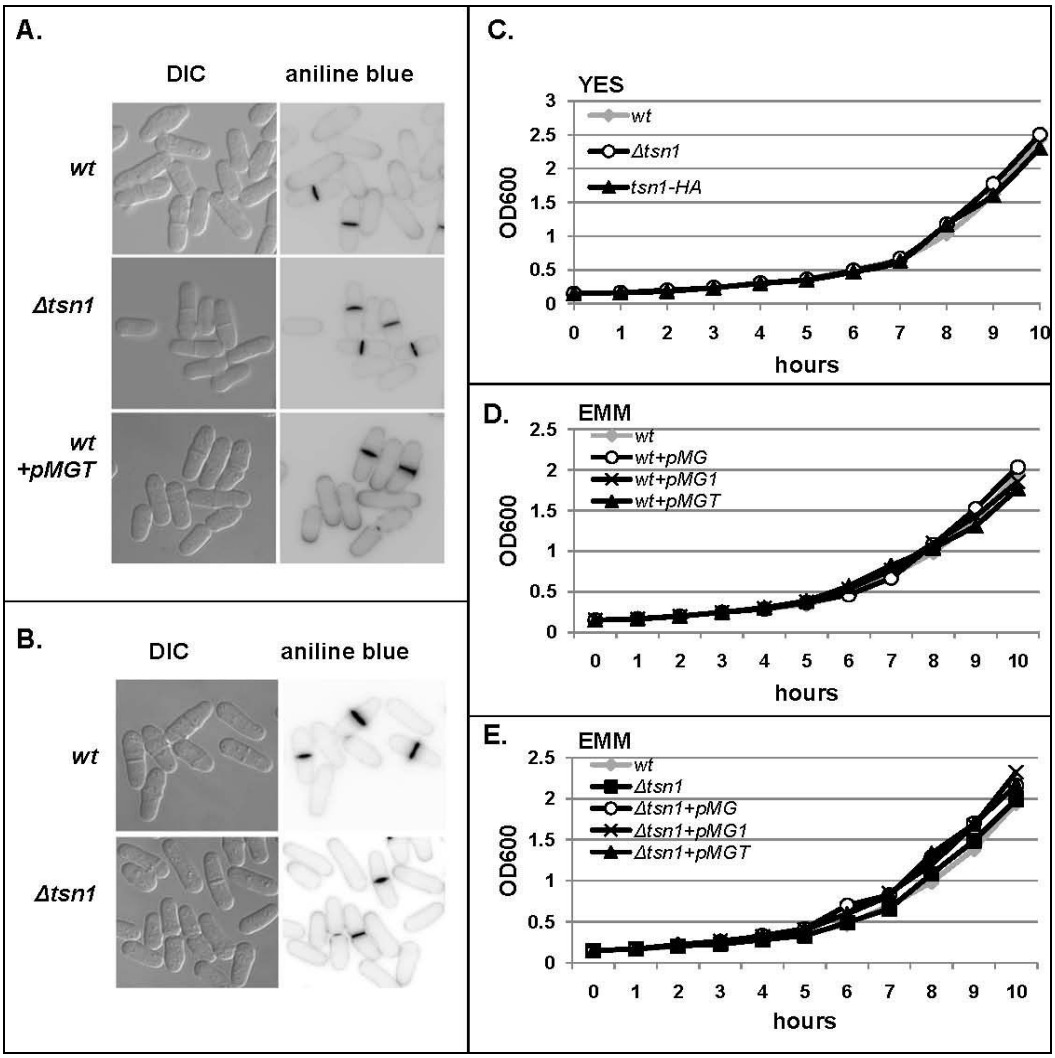

Figure. 3.20. Characterization of haploid *tsn1* deletion and overexpression strains

A. DIC and fluorescent microscopy pictures comparing morphology of the exponentially growing haploid wild type cells, *Δtsn1(nat)*) cells and *wt + pMGT* cells in minimal EMM medium under the conditions when *nmt1*$^{+}$ promoter is on. **B.** The same as A, but haploid wild type cells and *Δtsn1(nat)* cells in rich YES medium are compared. **C., D., E.** Growth curves comparing the growth rates of *wt, tsn1-HA* and *Δtsn1(nat)* strains in YES medium (C.) and growth curves comparing the growth rates of wild type cells (D.) or *Δtsn1(nat)* cells (E.) transformed with empty pMG plasmid, pMG1 plasmid and pMGT plasmid in the EMM medium at 30°C.

3.3.2. Both overexpression and deletion of *tsn1+* decrease the rate of meiotic differentiation

The amount of *tsn1* and *rct1* RNAs significantly increases during the meiosis I and II (Mata et al., 2002), pointing at the possible role of these two proteins in the regulation of meiotic differentiation. Furthermore, the expression of Rct1 protein seems to be an important factor for the correct meiotic differentiation and the meiosis-specific splicing of certain genes (Gullerova et al., 2007). Therefore I decided to investigate a possibility that the expression of Tsn1 protein is also important for the correct progression of meiosis.

First, I generated stable *Δtsn1(nat)/Δtsn1(kan)* diploid strain by mating *Δtsn1(nat)* and *Δtsn1(kan)* haploid strains and compared its sporulation rate with the sporulation rates of the diploid wild type and heterozygous *rct1/Δrct1* strains. To assay for the sporulation rates I induced meiosis in the nonsynchronous cell culture, simply by shifting the cells from the rich YES medium to the minimal medium without nitrogen (EMM-NA), and counted the number of asci generated after 22 hours at 28°C. Under these conditions 29,7% of wild type cells and 58,4% of *rct1/Δrct1* cells were already sporulated, but only 9,7% of *Δtsn1(nat)/Δtsn1(kan)* cells generated spores (Fig. 3.21.A.). The increased rate of sporulation in *rct1/Δrct1* cells is well consistent with the already published data (Gullerova et al., 2007), whereas the decreased sporulation of *Δtsn1(nat)/Δtsn1(kan)* has not yet been reported. In parallel with the experiment described above I performed the sporulation assays with the diploid wild type cells overexpressiong Rct1-HA (*2n wt + pMG1*) and Tsn1-HA (*2n wt + pMGT*) strains. Under the same conditions as above the

overexpression of Rct1-HA protein (*2n wt + pMG1)* resulted in decreased sporulation efficiency (3,9% of cells were sporulated, Fig. 3.21.A.). Similarly, the overexpression of Tsn1-HA protein also resulted in decreased sporulation efficiency (9,4% of cells were sporulated, Fig. 3.21.A.). From these experiments I concluded that the precise amount of Tsn1 protein is important for the efficient meiotic differentiation, because both, the increased and the decreased level of the Tsn1 protein expression, resulted in the decreased rate of spore formation.

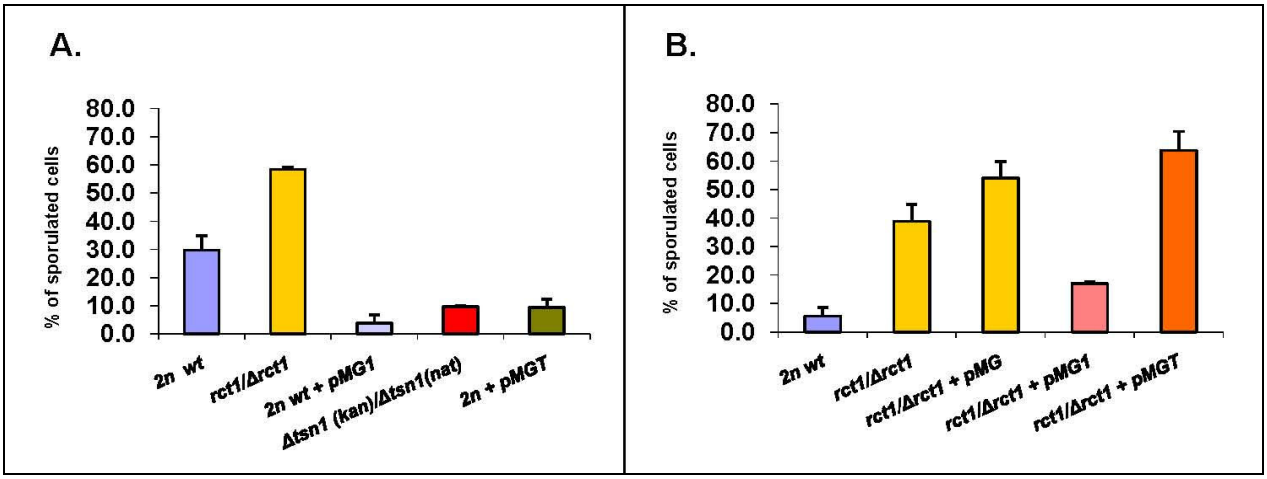

Figure 3.21. The *tsn1* and *rct1* genes are required for proper meiotic progression

A. Results of the sporulation assays comparing the sporulation efficiency of diploid wild type strain, *rct1/Δrct1* strain, *Δtsn1(nat)/Δtsn1(kan)* strain and diploid wild type strains overexpressing Rct1-HA (*2n wt + pMG1*) or Tsn1-HA (*2n wt + pMGT*) proteins. Bars in the graph represent the percentage of cells sporulated after 22 hours at 30 °C under the conditions of nitrogen starvation (EMM-NA, 1% glucose). **B.** Graph comparing sporulation efficiency of *rct1/Δrct1* strain with strains transformed either with empty pMG plasmid (*rct1/Δrct1 + pMG*) or pMG1 plasmid overexpressing Rct1-HA protein (*rct1/Δrct1 + pMG1*) or pMGT plasmid overexpressing Tsn1-HA protein (*rct1/Δrct1 + pMGT*). Bars in the graph represent the percentage of cells sporulated after 20 hours at 28 °C under the conditions of nitrogen starvation (EMM-NA, 1% glucose). The error bars represent standard deviation based on three independent biological experiments.

The Rct1 and Tsn1 proteins might act either together at a specific stage of meiotic differentiation, or independently of each other. I therefore asked, whether the overexpression of Tsn1-HA from pMGT plasmid can complement the increased sporulation of *rct1/Δrct1* cells. As can be seen in Fig. 3.21.B., the enhanced sporulation of *rct1/Δrct1* strain or *rct1/Δrct1 + pMG* strain (38,8% and 54% of cells were sporulated) was complemented by the overexpression of Rct1-HA protein from pMG1 plasmid (17% of cells were sporulated), but not by the overexpression of Tsn1-HA protein from pMGT plasmid (63,7% of cells were sporulated). This experiment shows that the deletion of one allele of *rct1* gene in the *rct1/Δrct1* strain is epistatic over the *tsn1* overexpression and suggests that the Rct1 has a function in meiotic differentiation that is superior to the function of Tsn1.

The *rct1/Δrct1* asci were reported to harbor a distinct morphology; being shorter and more compact than wild type asci (Gullerova et al., 2007). By contrast, the *Δtsn1(nat)/ Δtsn1(kan)* asci were linear like the wild type asci, but the spores in the asci seemed to be only loosely packed when compared to the wild type spores (Fig. 3.22.B.). Nevertheless, the statistical analysis did not reveal any significant differences in the size of asci between the analyzed strains, except of the shorter *rct1/Δrct1* asci (Fig. 3.22.A.). Interestingly, the reduced size of *rct1/Δrct1* asci was not complemented by Rct1 overexpression from pMG1 plasmid, but partially complemented by the overexpression of Tsn1 from pMGT plasmid (Fig. 3.22.A.).

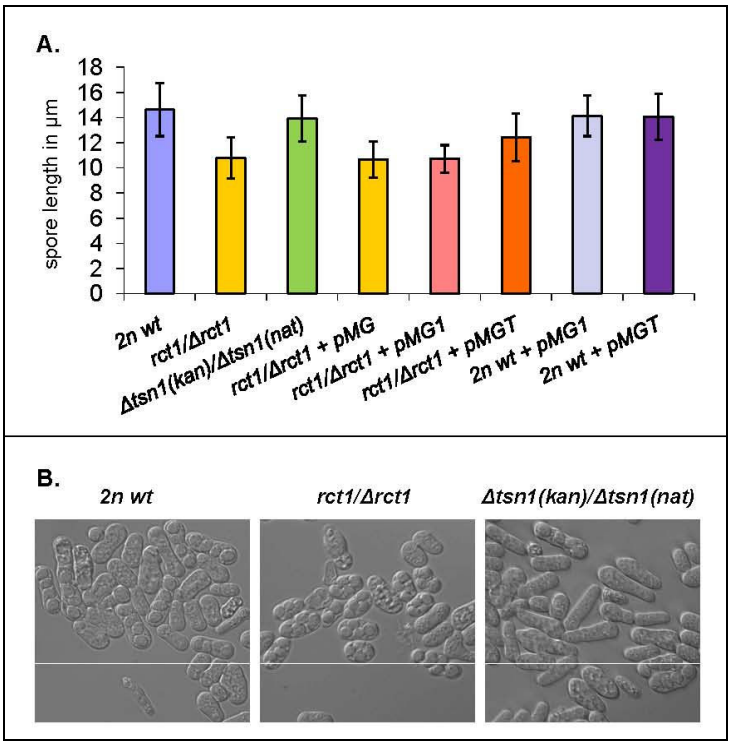

Figure 3.22. Morphology of acsi from the sporulation assays

A. Statistical analysis of the length of asci from the sporulation assays in Fig. 3.21. The length was measured between the most distant points of the ascus using the ImageJ software. **B.** DIC picture of measured spores, showing the normal morphology of *2n wt* asci, shorter and compact morphology of *rct1/Δrct1* asci and normal morphology of *Δtsn1(nat)/ Δtsn1(kan)* asci.

3.3.3. Expression of Tsn1 protein is increased during meiosis I and II

In the previous section I showed that the deletion of *tsn1* gene in diploid cells results in reduced meiotic efficiency. Furthermore, it was reported previously that the expression of *tsn1* mRNA is upregulated in middle meiosis (Mata et al., 2002). Therefore, I decided to determine the stage of meiotic differentiation at which the Tsn1 protein acts. I took the advantage of the *pat1-114* mutation that allows to synchronize the diploid *pat1-114/pat1-114* cells early in the meiosis by nitrogen starvation, and constructed *tsn1-HA/tsn1-HA pat1-114/pat1-114* diploid strain expressing wild type levels of the HA-

tagged Tsn1 protein. I synchronized these cells by the nitrogen starvation, induced them for meiosis by a temperature shift from 25°C to 34°C and sampled the cell culture at indicated time points (see Fig. 3.23.). First, I analyzed the total protein extracts of the samples from individual time points on the Western blots with anti-HA antibodies to reveal the changes in Tsn1-HA expression in the time course of meiosis. As shown in Figure 3.23., the Tsn1-HA specific signal increases 2 to 3 hours after the induction of meiosis and decreases approximately 6 hours after the induction of meiosis, being highest between the 3rd and 5th hour. As control Western blots with anti-tubulin antibodies did not show any differences between time points this change seem to be specific.

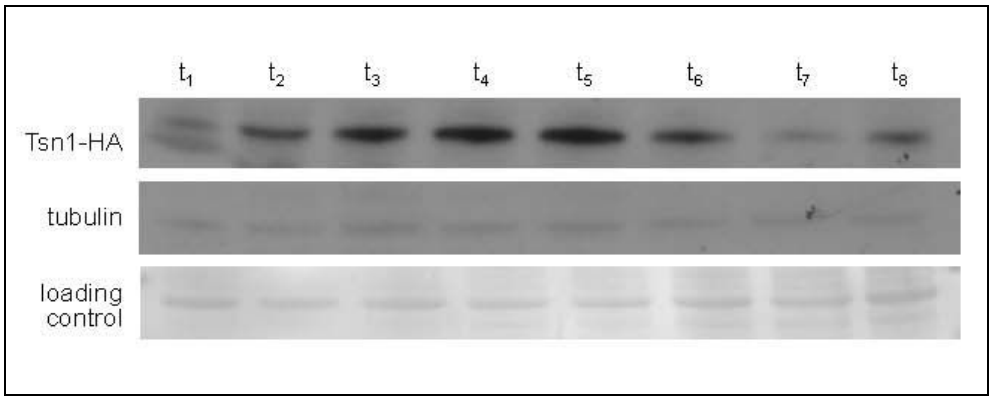

Figure 3.23. Tsn1 expression is increased during meiosis I and II of meiotic differentiation

Western blots with anti-HA antibodies (top) and with anti-tubulin antibodies (middle) comparing the expression of Tsn1-HA protein and tubulin in the time course of *tsn1-HA/tsn1-HA pat1-114/pat1-114* synchronous meiosis. The equal amounts of total proteins were loaded per lane (in µg). At the bottom is a section of the ponceau stained membrane showing the equal loading of protein extracts. The individual time points (t_1 to t_8) correspond to hours after the induction of meiosis by temperature shift.

To determine the stage of meiosis at which the expression of Tsn1-HA is increased, I counted the percentage of cells with one, two or four nuclei at each time point and plot the values against the time course of the experiment. Figure. 3.24.A. shows that the highest Tsn1-HA signal roughly correlates with the first and second meiotic divisions.

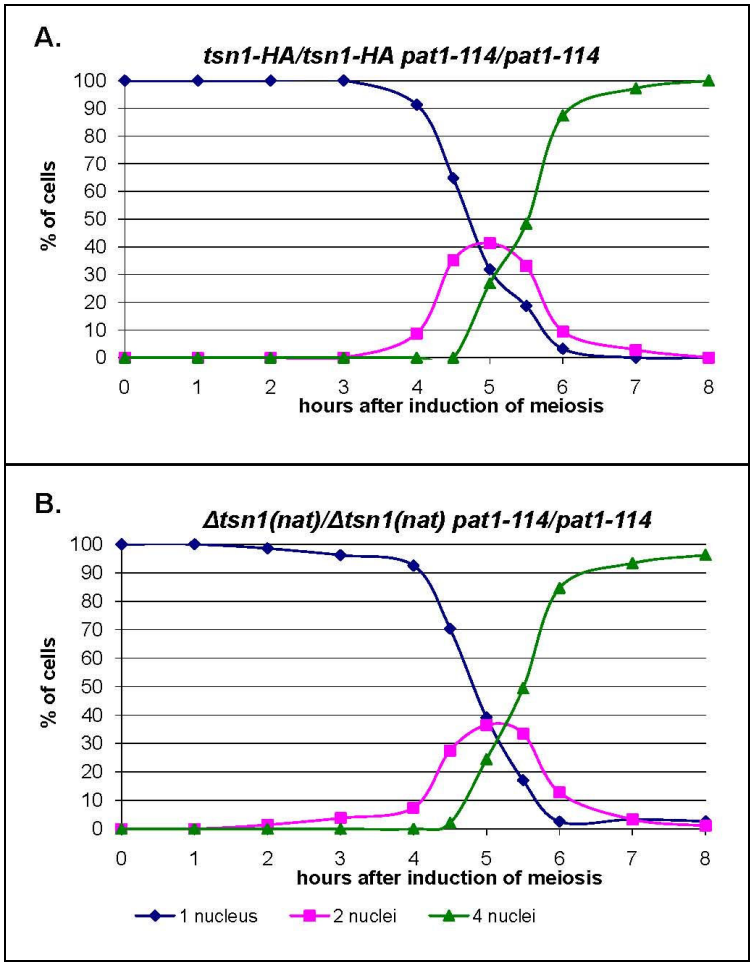

Figure 3.24. Deletion of *tsn1* gene in *pat1-114/pat1-114* genetic background does not have any effect upon the meiotic progression

Graphs showing the progression of *tsn1-HA/tsn1-HA, pat1-114/pat1-114* (**A.**) and *Δtsn1(nat)/Δtsn1(nat) pat1-114/pat1-114* (**B.**) strains in the time course of the synchronous meiosis. The progression was monitored by counting the cells with one nucleus (diamonds), two nuclei (squares) and four nuclei (triangles) in Hoechst stained samples. The samples were taken at the indicated time points after the induction of meiosis by temperature shift.

The experiments with the *Δtsn1(nat)/Δtsn1(kan)* strain described in part 3.3.2. resulted in the decreased rate of meiotic differentiation in the non-synchronous meiosis (Fig. 3.21.A.). To find the particular stage of meiosis at which these cells slow down their differentiation I created *Δtsn1(nat)/Δtsn1(nat) pat1-114/pat1-114* strain suitable for the synchronous meiosis. I synchronized these cells as described above and followed their progression through meiosis, either by counting the percentage of cells with one, two or four nuclei at each time point or by measuring the DNA content by flow cytometry (not shown). Surprisingly, I found no difference in the differentiation of the control *tsn1-HA/tsn1-HA pat1-114/pat1-114* strain and the deletion *Δtsn1(nat)/Δtsn1(nat) pat1-114/pat1-114* strain, neither in the rate of sporulation, nor in the DNA content changes (Fig. 3.24.B.). One possible explanation is that the *pat1-114/pat1-114* genetic background interferes with the *Δtsn1(nat)/Δtsn1(nat)* phenotype. The other possible explanation for this discreepancy is that the genetic manipulations that were required to create *Δtsn1(nat)/Δtsn1(nat) pat1-114/pat1-114* strain had an unknown suprressory effect over the *tsn1* deletion.

3.3.4. Is Tsn1 a nuclear protein?

In fission yeast translin was reported to localize predominantly to the nucleus and only partially to the cytoplasm of the cells overexpressing Tsn1 from *nmt1*$^{+}$ promoter (Matsuyama et al., 2006). However, this report is based on the data from a large scale screen, and therefore I attempted to investigate the Tsn1 localization in the more focused experiments. First, I attempted to tag *tsn1*$^{+}$ gene with the GFP protein coding sequence directly on the chromosome to obtain a strain expressing wild type levels of

fluorescently labeled Tsn1 protein. For this purpose I amplified *GFP::kanMX* region from pSMRG2 + GFP5 plasmid and recombined it downstream of *tsn1* ORF. By this approach I obtained the strain with *GFP::kanMX* sequence incorporated at the expected location; however, I was neither able to detect the Tsn1-GFP protein on the Western blots with anti-GFP antibodies, nor did I detect the GFP fluorescence under the fluorescent microscope (not shown). I consider it likely that the GFP tag renders the Tsn1 more prone for degradation, and therefore undetectable. Alternatively, the GFP tag might interfere with the splicing of *tsn1* gene and as a result no functional protein is generated. The lack of the Tsn1-GFP protein in the cells tagged with the GFP coding sequence on the chromosome might also be a specific effect of GFP tag, and could possibly be overcome by using another fluorescent protein tag. Therefore, I decided to tag *tsn1* gene with the tdTomato fluorescent protein, a dimmer of the Cherry protein, with a different structure than the GFP. However, I again failed to obtain the strain expressing Tsn1-tdTomato detectable on the Western blots with anti-dsRed antibodies or under the microscope (not shown).

Because I could not obtain the strain expressing fluorescently labeled Tsn1 protein from *tsn1*$^{+}$ native promotor, I have decided to assay for Tsn1-HA localization in the haploid wild type cells overexpressing Tsn1-HA from pMGT plasmid. For this purpose I fixed exponentially growing cells in EMM medium with formaldehyde and immunostained them with anti-HA antibodies and ALEXA 568 coupled secondary antibodies. In parallel I processed the haploid wild type cells overexpressing Rct1-HA from pMG1 plasmid as a

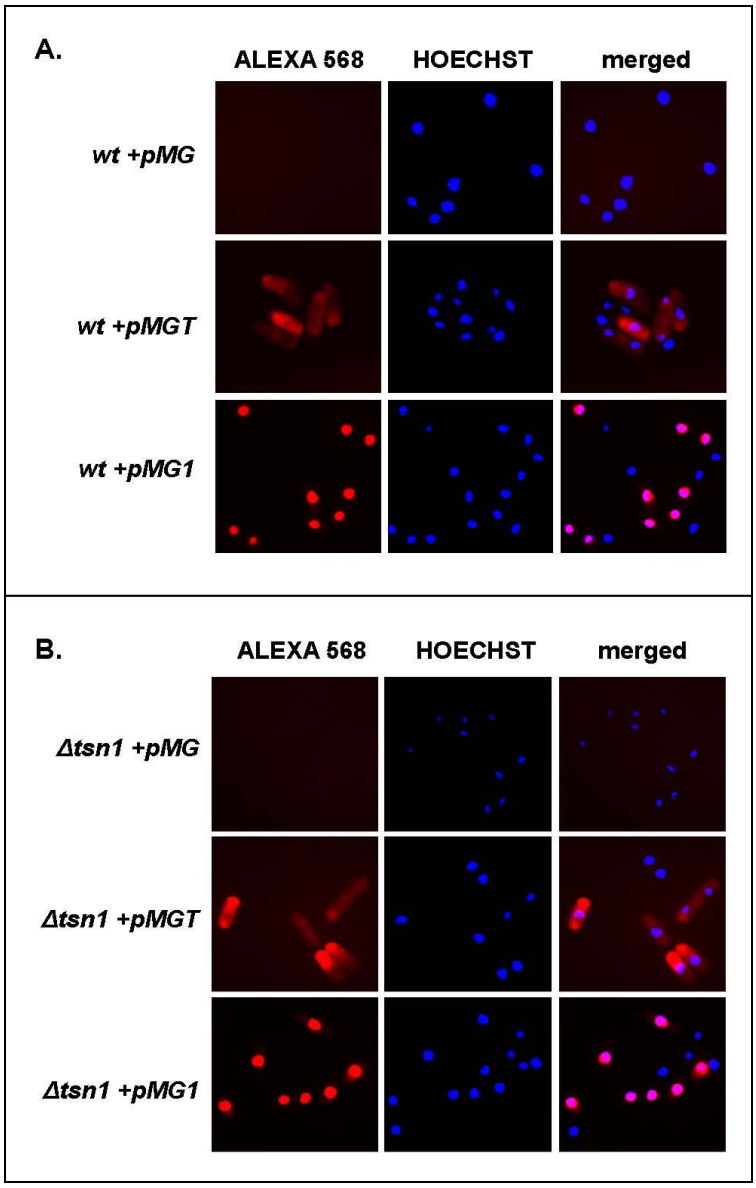

Figure 3.25. Immunolocalization of Tsn1 and Rct1 proteins in haploid cells

Fluorescent micrographs of immunostained Tsn1-HA and Rct1-HA proteins in the formaldehyde fixed cells. The cells were probed with anti-HA antibodies and Alexa 568 conjugated secondary antibodies to stain HA-tagged proteins and with Hoechst to stain DNA. Pictures on the left show Alexa 568 specific signal, in the middle Hoechst specific signal and on the right are the merged pictures. **A.** Immunolacalization of Tsn1-HA and Rct1-HA proteins in haploid wild type cells transformed with either empty pMG plasmid (*wt + pMG*) expressing no HA-tagged protein or with pMGT plasmid (*wt + pMGT*) overexpressing Tsn1-HA protein or with pMG1 plasmid (*wt + pMG1*) overexpressing Rct1-HA protein. Cells were grown in EMM medium to allow expression from the *nmt1*$^{+}$ promotor. **B.** Same as A, but in *Δtsn1(nat)* genetic background.

positive control and the haploid wild type cell transformed with empty pMG plasmid as a negative control. To visualize the localization of nucleus the DNA was stained with Hoechst dye. As can be seen in Figure 3.25.A (bottom), I found the Rct1-HA specific signal to co-localize with the DNA signal at the cell nucleus as reported previously (Gullerova et al, 2007). By contrast, the Tsn1-HA specific signal was rather evenly distributed throughout the cell without any predominant localization in any specific cellular compartment (Fig. 3.25.A., middle). However, I could not exclude that a small portion of the protein is indeed localized to the nucleus. Because the untagged Tsn1 protein expressed from its native promoter might interfere with the Tsn1-HA protein localization in the wild type cells, I also immunostained the haploid *Δtsn1(nat)* cells overexpressing Tsn1-HA and Rct1-HA proteins from pMGT and pMG1 plasmids respectively. However, the patterns of Rct1-HA and Tsn1-HA localization were the same as in the wild type background (Fig. 3.25.B.). Similar results were obtained with the diploid wild type strains transformed with pMG and pMGT plasmids (Fig. 3. 26.).

The overexpression of a protein might result in its misslocalization in the cell. Therefore I decided to repeat the immunostaining experiment with a strain expressing Tsn1-HA protein from its native promoter on the chromosome. For this purpose I amplified the *tsn1::HA::kanMX* region from pMGT plasmid and recombined it downstream of $tsn1^{+}$ promoter so that the entire $tsn1^{+}$ ORF was replaced. After backcrossing I obtained a stable strain expressing HA-tagged protein that was proved to be the *tsn1-HA* by PCR genotyping, sequencing and Western blotting. I immunostained the strain with anti-HA antibodies and ALEXA 568 coupled secondary antibodies, as already described above, but

the signal obtained was repeatedly to low to be detected under the fluorescence microscope (data not shown). In my experience the FLAG tag immunostaining with anti-FLAG antibodies gives better signal then the HA tag immunostaining with anti-HA antibodies, when the low expressed proteins are immunostained. Therefore, I tried to tag the *tsn1*$^{+}$ with the *FLAG::hphMX* sequence directly on the chromosome, however, I failed to obtain any strain expressing the Tsn1-FLAG protein by this approach (data not shown). Furthermore, I also attempted to determine Tsn1-HA localization in *tsn1-HA/tsn1-HA, pat1-114/pat1-114* cells undergoing synchronous meiosis, but I was not able to detect any Tsn1-HA specific signal in these cells as well (data not shown).

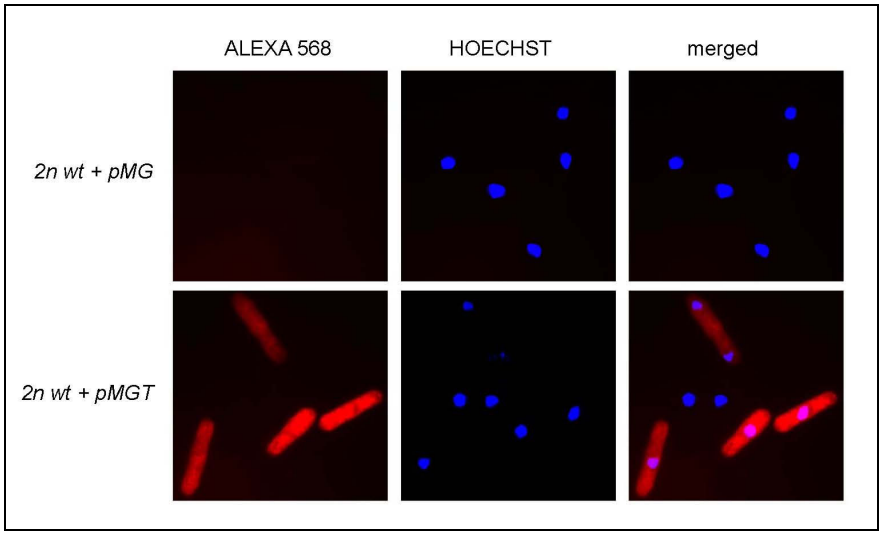

Figure 3.26. Immunolocalization of Tsn1 protein in diploid cells

Fluorescent micrographs of immunostained Tsn1-HA protein in the formaldehyde fixed *2n + pMG* and *2n + pMGT* cells. Cells were grown in EMM medium to allow expression from the *nmt1*$^{+}$ promoter and probed with anti-HA antibodies and Alexa 568 conjugated secondary antibodies to stain HA-tagged proteins and with Hoechst to stain DNA. Pictures on the left show Alexa 568 specific signal, in the middle Hoechst specific signal and on the right are the merged pictures.

3.3.5. Tsn1 is associated with transcriptionally active chromatin

From the experiments above is not clear whether the Tsn1 is a nuclear protein or not, however, it is possible that a small portion of Tsn1 is also present in the nucleus. Because I originally identified Tsn1 protein as the interacting partner of Rct1, which is known to associate with the transcriptionally active chromatin, I asked whether at least a portion of Tsn1 protein in the cells could be possibly associated with the transcriptionally active chromatin as well. For this purpose I performed the Tsn1-ChIP experiments with anti-HA antibodies and formaldehyde cross-linked *tsn1-HA* cells. Precipitated DNA was amplified with the oligonucleotides covering *dis2* and *rho1* transcriptional units. The results clearly show that the Tsn1-HA protein is significantly enriched along these transcriptional units, when compared to the background signal obtained in the parallel experiments without the antibodies (Fig. 3.27.). Again, the distribution of Tsn1-HA protein along these genes closely resembled that of RNAPII and Rct1-HA (compare Fig. 3.27. with Fig. 3.14 and Fig. 3.15.), suggesting that Tsn1 could associate with RNAPII and Rct1 along the transcribed genes.

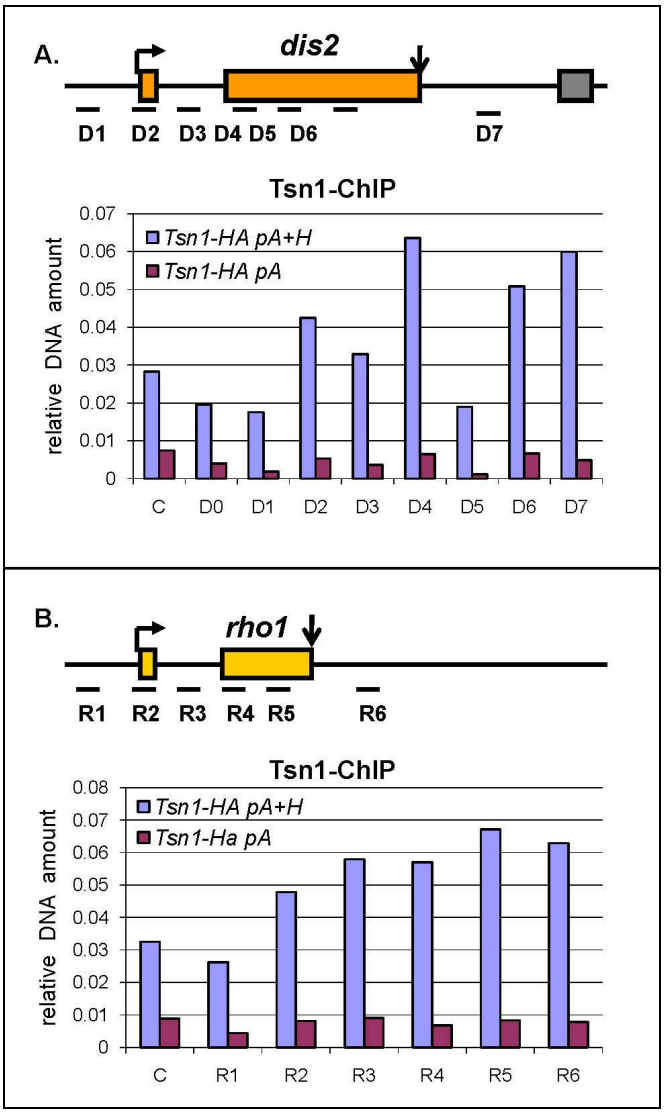

Figure 3.27. Tsn1 protein is associated with chromatin

A., B. Graphical representation of Tsn1-ChIP experiment results showing the occupancy of Tsn1-HA along *dis2* (A.) and *rho1* (B.) transcriptional units. Shown are the relative values of the DNA amount precipitated with anti-HA antibodies (pA+H). For comparison the relative DNA values obtained in the parallel experiment without the antibody (pA) are shown. All relative DNA values were normalized to the signal obtained at an unrelated rDNA region (C). Schematic representations are as in the Fig. 3.14. One of three independent biological experiments is shown.

4. DISCUSSION

4.1. ROLE OF Rct1 IN COUPLING pre-mRNA SPLICING AND TRANSCRIPTION

Rct1 is a protein with three distinct domains: the PPIase, the RMM and the RS domain. The PPIase domain is characteristic of peptidyl prolyl isomerases (PPIases) (see part 1.5. for details), whereas the RRM and RS domains are characteristic of SR proteins (see part 1.3. for details). Rct1 is an essential protein with a role in the regulation of RNAPII transcription (Gullerova et al., 2007). It has been reported to co-immunopreciptate with the phosphorylated CTD of RNAPII and to modulate the CTD phosphorylation and RNAPII transcription *in vivo* (Gullerova et al., 2007). The interaction between the Rct1 and CTD is mediated by the PPIase domain of Rct1, while the RRM and RS domains are negligible for the interaction (Skrahina T, 2009). Rct1 also interacts *in vitro* with the Ser2-specific CTD kinases Cdk9 and Lsk1, but does not interact with the Ser5-specific CTD kinases Msc6 and Srb10 and CTD-specific phosphatases Fcp1, Scp1 and Ssu72 (Skrahina T, 2009). Again the PPIase domain is responsible for these interactions, as well as for the inhibition of Cdk9 kinase activity on CTD substrate (Skrahina T, 2009). The plant homologue of Rct1 AtCyp59 regulates RNAPII CTD phosphorylation as well, and furthermore it also associates with the plant SR proteins (Gullerova et al., 2006). Here I investigated a possibility that Rct1 interacts with fission yeast SR proteins and couples splicing to RNAPII-mediated transcription.

4.1.1. Interactions of Rct1 with Srp1, Srp2, Prp2 and Dsk1 proteins and their possible consequences.

There are two characterized SR proteins in fission yeasts Srp1 and Srp2. The Srp2 is a nuclear protein with proposed role in the regulation of pre-mRNA splicing. On the contrary, the Srp1 is considered to be a shuttling protein without any known direct function in the pre-mRNA splicing (Lutzelberger et al., 1999, Tang et al., 1998, 2000, 2002, 2007, see also part 1.3.3. for details). I showed that Rct1 interacts with both fission yeast SR proteins Srp1 and Srp2, as well as with the SR protein-like splicing factor Prp2 () and the RS domain-specific kinase Dsk1 (Fig. 3.7.A.). All of these proteins either harbor an RS domain (Srp1, Srp2, Prp2) or have a strong preference for the RS domain as a substrate (Dsk1) (Tang et al., 1998, 2000, 2002, 2007). The RS domain is also absolutely required for the interactions between the AtCyp59 and the plant SR proteins (Gullerova et al., 2006). Therefore, I decided to test if the RS domains of Rct1, Srp1, Srp2 and Prp2 are needed for the interactions. The pull down assays with different deletion mutants revealed that indeed the RS domains of Rct1, Srp2 and Prp2 are involved in the intearctions (Fig 3.7.B and C.). Furthermore, I could show that the PPIase domain of Rct1 and RRM domain of Srp1 also contribute to the interactions.

Although several repetions of the pull-down experiment reproducibly revealed interaction of Rct1 with Srp1 and Srp2, I could not detect the same interactions in the co-immunoprecipitation assays (see Fig. 3.11.). Similarly, Das et al. (2007) failed to co-immunoprecipitate RNAPII using the antibodies against the mammalian SR proteins, but succeeded whit GST-SR protein fusions. As suggested by Das et al. (2007), it is possible

that the epitops recognized by SR protein-specific antibodies are not accessible in the native complexes. Another possible explanation is that the interactions detected in the pull down assays are only transient and therefore under detection limit of the co-immunoprecipitation assay. The interactions of different SR proteins are usually phosphorylation-dependent (Lorkovic et al., 2004). The phosphorylation state of a bacteria-produced SR protein is likely different form its native phosphorylation state and might also account for the different outcome of the pull down and immunoprecipitation assays.

The Dsk1 kinase interacted with Rct1 RS domain, but not with the PPIase or RRM domain of Rct1, in the pull down assays (Fig. 3.7.B and C.). This is well consistent with RS domain-specific kinase activity of Dsk1 on Srp1, Srp2 and Prp2 substrates (Tang et al., 1998, 2000, 2002, 2007) and opens a possibility that Rct1 RS domain is also phosphorylated by Dsk1. The phosphorylation of Srp2 and Prp2 RS domains by Dsk1 is important for correct nuclear localization of Srp2 and Prp2 proteins (Lutzelberger et al., 1999; Tang et al., 2007). Also the Rct1 RS domain seems to be important for correct nuclear localization of Rct1 protein (Lorkovic Z, unpublished data). It is therefore possible that Rct1 nuclear localization is regulated by Dsk1-dependent phosphorylation of Rct1 RS domain. However, the dephosphorylation assays revealed that whereas Srp1 and Srp2 exist in the cells predominantly in a highly phosphorylated form, the Rct1 is only modestly, if at all, phosphorylated (see Fig. 3.9.E.). Interestingly Dsk1 shuttles between the cytoplasm and nucleus in cell cycle-dependent manner and its activity is up-regulated by autophosphorylation (Takeuchi and Yanagida, 1993; Tang et al., 1998). Although it

accumulates in the cytoplasm from G1 to G2 phase of the cell cycle, it is most active in the M phase, when it accumulates in the nucleus (Takeuchi and Yanagida, 1993). Some phenotypes of Rct1 deletion mutants suggest that Rct1 is important for the correct cell cycle progression (Gullerova et al., 2007; Lorkovic ZJ, unpublished results). It is therefore tempting to speculate that Dsk1 might regulate the activity of Rct1 in a cell cycle-dependent manner.

The interaction of Rct1 with Srp2 requires the RS domain of Srp2 protein and both PPIase and RS domain of Rct1 at the same time (Fig. 3.7.B. and C.). Because the deletion of Srp2 RS domain fully abolished the interaction, the Srp2 RRM1 and RRM2 domains are likely not involved. Considering the RS domain of Srp2 to be the physiological substrate of Rct1, we can speculate that the PPIase domain is required to modulate the conformation of the Srp2 RS domain, whereas the RS domain might be needed to stabilize and specify the interaction. The Srp2 is known to bind the model ESEs (Webb et al., 2005b) and to interact with $U2AF^{SM}$ (Webb and Wise, 2004). The $U2AF^{SM}$ creates complex with Prp2 ($U2AF^{LG}$) and binds to the 3' splice site of an intron (Wentz-Hunter KPJ, 1996; Webb et al., 2005a). The regulation of Srp2 activity by Rct1 can therefore have a direct effect on the 3' splice site selection. Recently, it was shown that in mammals the RS domain of ESE bound SR protein might employ the RS domain to contact the RNA duplex created between the pre-mRNA and U2 snRNA and thus modulate the spliceosomal rearrangements (Shen et al., 2004b). Although there is no evidence that ESE bound Srp2 employs the RS domain for such interaction in fission yeasts, it is tempting to speculate

that Rct1 modulates the RNA-RNA interactions in the spliceosomal A complex by modifying the Srp2 RS domain.

The interaction of Rct1 with Srp1 requires only the RS domain of Rct1, but not the PPIase and RRM domain of Rct1, and does not require the Srp1 RS domain (3.7.B and C.). It is possible that the Rct1 RS domain interacts with the RRM domain of Srp1. However it is also posible that Rct1 interaction with Srp1 detected in the pull down assay is not direct, but mediated by another protein. This bridging protein could be Srp2, because it is known to employ the RRM1 domain for the interaction with Srp1 RMM (Lutzelberger et al., 1999; Tang et al., 2002) and RS domain for the interaction with Rct1 RS domain (Fig. 3.7.B.). Srp1 is a nuclear protein (see Fig. 3.10.); however, unlike to Srp2 and Rct1 it was neither cross-linked with the chromosomal DNA of *dis2* gene (see Fig. 3.18.), nor it interacted with RNAPII in the pull down assay (see Fig.3.7.A.). It is therefore plausible that the Srp1 nuclear function is distinct from the Srp2 function. As was proposed by Tang et al. (2007) Srp1 is likely imported into the nucleus in a complex with Srp2. Furthermore, Srp1 inhibits the ability of Srp2 to regulate splicing of *tfIId* pre-mRNA via Srp1 RRM interaction with Srp2 RRM2 (Gross et al., 1998; Lutzelberger et al., 1999). This suggests that the essential protein Srp2 must be released from Srp1/Srp2 complex before *tfIId* pre-mRNA is spliced, and therefore the likely function of Srp1 is to regualte the activity of Srp2 protein. However, Srp1 might have another, yet not determined, functions in gene expression.

The interaction between the Rct1 and Prp2 is particularly interesting, because it directly links Rct1 with the core splicing machinery. The Rct1 interacts with Prp2 via PPIase and RS domain independently of each other, but only the RS domain of Prp2 is required for the interaction (see Fig. 3.7.B. and C.). The Prp2 is an homologue of $U2AF^{65}$, a component of spliceosomal E and A complexes implicated in the 3' splice site selection (Potashkin et al., 1993; Valcarcel et al., 1996; Webb et al., 2005a; Behzadnia et al., 2007). In mammalian cells the RS domain of $U2AF^{65}$ subunit is required to promote transition from the spliceosomal E to A complex (Valcarcel et al., 1996). In agreement with this observation the deletion of Prp2 RS domain in *S. pombe* has dominant negative effect on pre-mRNA splicing (Webb et al., 2005a). Furthermore, Prp2 is also necessary for splicing of introns without the Py tract or introns with an atypical Py tract (Romfo et al., 1999, Sridharan and Singh, 2007) and tightly cooperates with $U2AF^{SM}$ in the 3' splice site selection (Webb et al., 2005a). It is therefore possible that the RS domain of Prp2 might act to promote the rearrangements between the spliceosomal E and A complexes in the fission yeasts as well (Webb et al., 2005a). If we assume that the RS domain of Prp2 is indeed the physiological target of Rct1, we can speculate that Rct1 is needed to regulate the substrate specificity of Prp2 RS domain *in vivo*. This can be provided either by changing the conformation of RS domain by PPIase activity of Rct1, or simply by the means of chaperoning protein-protein interactions. As a consequence, Rct1 could modulate the Prp2-dependent 3' splice site selection or the Prp2-dependent spliceosomal A complex assembly.

4.1.2. The potential role of Rct1 in coupling splicing with RNAPII transcription

The RNAPII transcription cycle has several stages that are controlled by CTD domain phosphorylation (Egloff and Murphy, 2008; Buratowsky S, 2009; see also part 1.1. for details). The proper control of every stage is required to assure the fidelity of RNAPII transcription. The passage from one stage to the other is controlled by complex signalling checkpoints that are also coupled to the processing of nascent pre-mRNA (Cramer et al., 2001; Phatnani and Greenleaf, 2006; Cho EJ, 2007; Egloff and Murphy, 2008; Margaritis and Holstege, 2008; Buratowski S, 2009; Cowling VH, 2010). Because Rct1 has a negative effect on Cdk9 kinase activity on Ser2 CTD substrate (Skrahina T, 2009), it might be required to regulate one or more RNAPII transcription checkpoints.

The genome wide ChIP analysis revealed that RNAPII distribution is different along the introns and exons. In mammals RNAPII signal is higher along the exonic than intronic sequences (Brodsky et al., 2005; Schwartz et al., 2009), wheras in *S. pombe* the RNAPII signal accumulates mainly along the intronic sequences of low expressed genes (Wilhelm et al., 2009). This suggests that the RNAPII processivity is reduced on introns in fission yeasts (further referred as an intron-dependent stalling of RNAPII), possibly as a consequence of a splicing quality control checkpoint. In accordance, my ChIP exepriments also show that the RNAPII signal is increased along the intronic sequences of low expressed *dis2* and *osm1* genes (see Fig. 3.14.A. and B.), but not along the highly transcribed *rho1* gene.

Rct1 is known to interact with CTD domain of RNAPII (Gullerova et al., 2007) and the distribution of Rct1 signal in Rct1-ChIP experiments along the *dis2, osm1* and *rho1* genes closely resemble that of RNAPII, suggesting that RNAPII and Rct1 ''travel'' along the genes together (compare Fig. 3.14 and 3.15.). Supported by an intriguing finding that the deletion of Rct1 RS domain changes the activity of GAL4-BD-Rct1 protein in the yeast two hybrid assay (Fig. 3.1.C.), I suggest that the RS domain of Rct1 might modulate the activity of full length Rct1 protein while associated with the RNAPII complex *in vivo.* Interestingly, the deletion of Rct1 RS domain also resulted in increased RNAPII signal on *dis2* transcriptional unit at the position D5, e.i. at the position where the signal is reduced compared to the intronic sequence in the full length protein background (Fig. 3.14.A.). Importantly, the deletion of Rct1 RS domain did not result in reduced or increased Rct1 protein levels along the *dis2*, *osm1* and *rho1* transcriptional units when compared to the full length Rct1 protein (Fig. 3.15.), although the total expression of Rct1 ΔRS protein was increased compared to the full length protein (see Fig. 3.12.D.). Therefore I propose that the Rct1 RS domain might be needed to release RNAPII from the intron-dependent stalling.

The inability of truncated Rct1 ΔRS protein to release the RNAPII from the intron depended RNAPII stalling on *dis2* gene can reflect (i) an impaired recruitment of splicing factors to transcribing RNAPII or (ii) an impaired spliceosome assembly or (iii) simply an impaired signalling between the spliceosome and RNAPII. I was able to show that Rct1 RS domain deletion does not affect the splicing efficiency of *dis2*, *osm1* and *rho1* mRNAs (Fig. 3.16 and 3.17.), arguing that the Rct1 RS domain deletion effect on the co-

transcriptional splicing is rather rate-limiting then qualitative trait. Furthermore, I showed that Srp2 protein, but not Srp1, associates with the *dis2*, *osm1* and *rho1* transcriptional units in ChIP experiments with similar distribution pattern as the RNAPII and Rct1. Consistent with the results of *in vitro* pull down experiments (Fig. 3.7.), this suggests that not only the Rct1, but also the Srp2 associates with RNAPII during the transcription. Furthermore, the deletion of Rct1 RS domain had no significant effect on Srp2 distribution along *dis2* transcriptional unit, showing that the recruitment of this splicing factor is not impaired.

It is not easy to reconstruct events that are needed to release RNAPII from the intron-dependent RNAPII stalling. The increased RNAPII signal on introns in fission yeasts was correlated with reduced H3K36me3 methylation signal (Wilhelm et al., 2009). It is therefore possible that the epigenetic marking contributes to the RNAPII release from intron-dependent stalling, as has already been suggested for some other organisms (Andersson et al., 2009; Schwartz et al., 2009; Hon et al., 2009; Kolasinska-Zwierz et al., 2009). My ChIP experiments suggest that yeasts require Rct1 for the RNAPII release from intron-dependent RNAPII stalling at *dis2* gene. Furthermore, my and some other ChIP data (Guiguen et al., 2007; Viladevall et al., 2009; Skrahina T, 2009) suggest that Cdk9, Rct1 and Srp2 are all associated with Ser5 and Ser2 phosphorylated RNAPII during the whole elongation phase of transcription. There is no similar experimental evidence showing that Prp2 is associated with RNAPII CTD during the whole elongation phase of transcription. But based on experiments with its mammalian homologue U2AF^{65} it seems plausible that it also associates with RNAPII, at least transiently (Robert et al., 2002;

Ujvari et al., 2004). Also my pull down experiments show that Prp2 is able to interact with the RNAPII *in vitro* (Fig. 3.7.A.). I consider it plausible that Cdk9, Rct1, Prp2 and Srp2 proteins interact transiently with each other on the RNAPII CTD platform and that the nature of these interactions changes in accordance with the progression of the splicing process. Therefore, I propose a following model for Prp2/Srp2-dependent Rct1-mediated RNAPII release from the intron dependent-stalling on *dis2* intron (Fig. 4.1.).

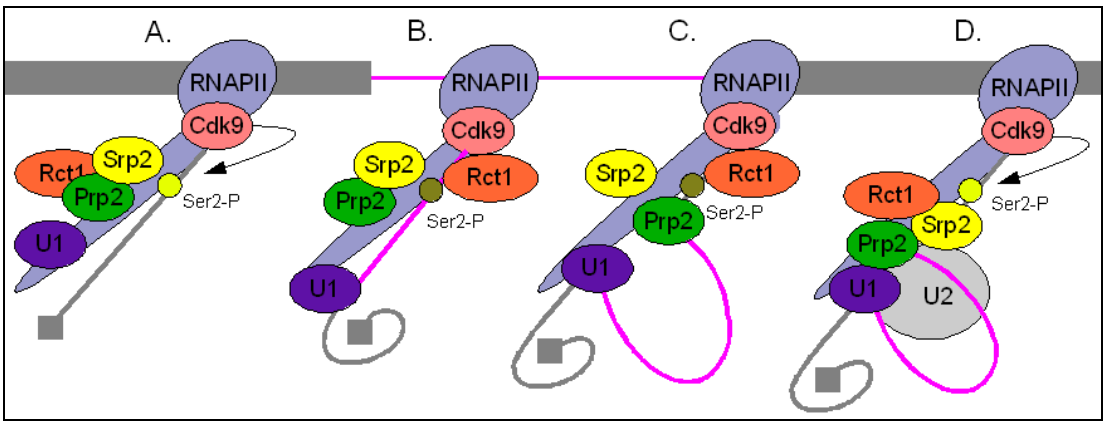

Figure 4.1. A proposed working model of Prp2 and/or Srp2-dependent Cdk9 inhibition by Rct1 on intron region.

During the productive elongation phase of transcription the Ser2 of RNAPII CTD is phosphorylated by Cdk9 kinase. The splicing factors (here U1 snRNP, Prp2 and Srp2) are pre-associated with the transcribing RNAPII, so that they are ready to recognize the signal sequences that appear on the nascent pre-mRNA (A). On the intron region Rct1 likely inhibits the activity of Cdk9 kinase that leads to reduced RNAPII processivity(B). At this stage the Prp2 and Srp2 are free to interact with the pre-mRNA. When the 5' and 3' splice site appear on the nascent pre-mRNA they are recognized by U1 snRNP and Prp2 and the spliceosomal E complex is created on RNAPII CTD platform (C). When an enhancer sequence appears on the nascent pre-mRNA it is recognized by Srp2 protein to stabilize the interactions between the U1 and U2 snRNPs of the spliceosomal A complex. Around this stage Rct1 likely dissociates from the inhibitory Cdk9 complex resulting in increased RNAPII processivity and in the RNAPII release form the intron-dependent stalling (D).

For simplicity, in my model Rct1 can interact either with Cdk9 or one of the splicing factors (Prp2 or Srp2) while on the RNAPII CTD platfom. It is plausible that Rct1 does not inhibit Cdk9 activity during the productive elongation on exon (Fig. 4.1.A.), because it would likely have a negative effect on RNAPII processivity (Gullerova et al., 2007; Skrahina T, 2009). It is possible that at this stage Rct1 also interacts with some other RNAPII associated factors, for example Srp2 and/or Prp2, or with the CTD itself. When RNAPII starts transcription of the intron, the Rct1 might create an inhibitory complex with Cdk9 that results in reduced RNAPII processivity (Fig. 4.1.B. and C.). But the reasons for reduced RNAPII activity might be of a complex character. For example, the overall chromatin architecture of intronic DNA might contribute to the reduced RNAPII processivity (Schwartz et al., 2009). However, there exists also experimental evidence suggesting that the spliceosomal assembly is coupled to the regulation of RNAPII processivity (Fong and Zhou, 2001; Kameoka et al., 2004; Bres etal., 2005; Barboric et al., 2009; see also part 1.4.1. for details). It is therefore possible that RNAPII elongation factors somehow sense the stage of spliceosomal assembly and adopt the RNAPII processivity and chromain architecture accordingly (Zhong et al., 2009). I have shown that Rct1 RS domain is required for RNAPII release from intron-dependent staling at *dis2* gene. I therefore consider it plausible that the Rct1 associated with Cdk9 somehow senses the progress of spliceosomal assembly, possibly by its RS domain and modulates the activity of Cdk9 accordingly. Other possible model scenario is that Rct1 is released from the interaction with Cdk9 and associates with Prp2 and/or Srp2 during a specific

step of spliceosomal assembly, e.g. spliceosomal A complex, resulting in enhanced RNAPII processivity (Fig. 4.1.D).

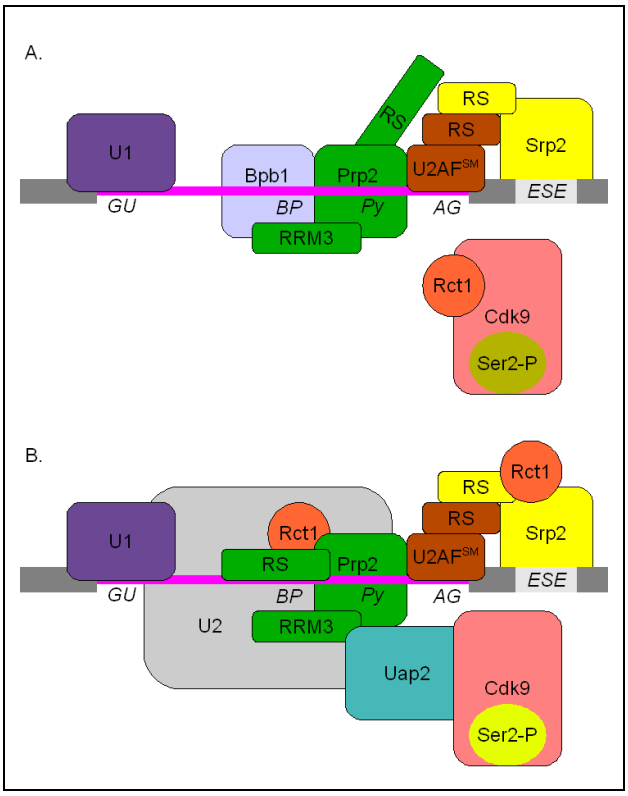

Figure 4.2. A proposed role for Rct1 in coupling spliceosomal assembly to RNAPII transcription in *S. pombe*.

In the pre-spliceosomal E complex the 5' splice site (GU) is recognized by U1 snRNP, the 3' splice site (AG) by $U2AF^{SM}$, the polypirimidine tract (Py) by Prp2 ($U2AF^{LG}$) and the branch point sequence (BP) by Bpb1 (SF1/BBP). The interaction between the $U2AF^{SM}$ and 3' splice site can be further stabilized by ESE-bound SR protein Srp2. At this stage Rct1 likely inhibits the activity of Cdk9 towards Ser2 of RNAPII CTD (A). In the pre-spliceosomal A complex the Bpb1 is replaced by U2 snRNP that interacts with U1 snRNP and Prp2. At this stage Prp2 might interact with Uap2 (TAT/SF1) that can positively stimulate the activity of CDK9 on Ser2 CTD substrate. At the same time Rct1 likely dissociates off the inhibitory Cdk9 complex and might modulate the activity and substrate specificity of Prp2 and/or Srp2 and therefore contribute to the spliceosomal rearrangements (B).

It is also possible that Rct1 changes the activity of Prp2 and/or Srp2 in a way that results in enhanced RNAPII transcription (Fig. 4.2.B.). For example, Prp2 is known to interact with Uap2, a fission yeast homologue of TAT-SF1 transcription factor (McKinney et al., 1997) and TAT-SF1 in turn interacts with CDK9/cyclin T1 complex (Fong and Zhou, 2001). The TAT-SF1 is a component of the mammalian spliceosomal A complex (Behzadnia et al., 2007) and is required for recruitment of snRNP complexes to RNAPII CTD (Fong and Zhou, 2001). There is no direct experimental evidence that TAT-SF1 stimulates the activity of CDK9 kinase; however, it seems plausible, because the snRNP recruitment is CTD phosphorylation-dependent (Fong and Zhou, 2001). It is therefore tempting to speculate that Rct1 might enhance the Cdk9 activity and RNAPII processivity in Prp2 and Uap2-dependent manner during the release from the intron dependent stalling (Fig. 4.2.B.).

It is also possible that different models are employed under different physiological conditions or on different genes. Indeed, the effect of Rct1 on RNAPII transcription and pre-mRNA splicing is likely gene-specific. Gullerova et al. (2007) showed that reduced Rct1 protein levels in diploid cells during the meiotic differentiation result in increased transcription and splicing of a subset of genes, which are specifically spliced upon the meiotic induction (Averbeck et al., 2005; Moldon et al., 2008). By contrast, the constitutively spliced genes are not influenced by Rct1 protein levels. Transcription-dependent splicing of one of these genes, *rem1*, is known to be regulated by the promotor sequence of the gene in cooperation with forkhead transcription factors Fkh1 and Mei4 in transcription-dependent manner (Malapeira et al., 2005; Moldon et al.,

2008; see also part 1.4.3. for details). Importantly, Moldon et al. (2008) showed that Mei4 is also required to load active spliceosome on transcribing RNAPII. Gullerova et al. (2007) showed that in the time course of meiosis Rct1 becomes enriched along the transcription units of Rct1-dependent genes. In combination with our current data, we can speculate that Rct1 might act to enhance Mei4-dependent spliceosomal recruitment along these genes, possibly via interactions with Prp2 and/or Srp2 and/or Srp1.

The gene specific effect of Rct1 on transcription and splicing likely occurs also in mitotic cells. For example the loss of Rct1 protein in cycling cells results in chromosome segregation and cytokinesis defects (Gullerova et al., 2007), suggesting that Rct1 effect on transcription is not general, but rather gene specific. Interestingly, similar phenotypes were assigned to mutations in Prp2, Cdk9 and Lsk1 proteins that are known to interact with Rct1 (Takahashi et al., 1994; Karagiannis et al., 2005; Webb et al., 2005a; Karagiannis and Balasubramanian, 2007; Viladevall et al., 2009). It is therefore tempting to speculate that Rct1 might regulate the expression and/or splicing of some genes required for chromosome segregation and cytokinesis.

4.2. INTERACTIONS OF Rct1 WITH TRANSLIN

The PPIases usually recognize and modify high number of protein targets. This is well documented in the case of Pin1/Ess1 PPIase that modulates the activity of many protein targets involved in diverse cellular processes (Lu and Zhou, 2007b). To extend our knowledge of Rct1 protein targets I performed yeast two hybrid screen with Rct1 as bait. By screening the *S. pombe* pTN-TH7 library of pooled mitotic and meiotic cDNAs I have identified several putative targets of Rct1 (Tab. 3.1.). Surprisingly, I did not identify any of the known Rct1 targets in the screen. This was particularly surprising, because the interactions between the AtCyp59 and plant SR proteins were detected employing similar yeast two hybrid assay (Gullerova et al., 2006). On the contrary, Rct1 interactions with CTD, Cdk9 and Lsk1 were studied solely *in vitro*, as any attempts to assay for the interactions in the yeast two hybrid assays were not successful (Skrahina T, 2009). One of the isolated cDNAs encoded a conserved protein translin (Tsn1). Based on our knowledge of Rct1 and available information about Tsn1 I decided to analyze Tsn1 interaction with Rct1 further.

Tsn1 is reported to be, like Rct1, a nuclear protein (ORFeome data in Matsuyama et al., 2006). Several reports suggests that Tsn1 is involved in RNA and/or DNA metabolism (Sengupta and Rao, 2002; Yang et al., 2003; Cho et al., 2005; Chiaruttini et al., 2009; Liu et al., 2009) and *tsn1* mRNA expression is up-regulated during meiotic differentiation in a similar manner to *rct1* mRNA (Mata et al., 2002). Furthermore, Tsn1 is highly conserved in higher eukaryotes, but like Rct1 does not have any homologue in *S. cerevisiae*.

Translin has been extensively studied in humans, mouse, fruit flies and also in fission yeasts and was implicated in diverse biological processes (reviewed in Li et al., 2008; Jaendling and McFarlane, 2010). These include mRNA trafficking and translational repression in mammalian neuronal cells (Gu et al., 1998; Muramatsu et al., 1998; Finkenstadt et al., 2000; Chiaruttini et al., 2009) and spermatocytes (Gu et al., 1998; Wu and Hecht, 2000; Yang et al., 2003; Cho et al., 2005), RISC-dependent mRNA degradation in *Drosophila* (Wang et al., 2004; Liu et al., 2009), regulation of steroidogenic factor-1 (SF-1)-stimulated transcription in mammals (Mellon et al., 2007), genome stability regulation and DNA recombination (Aoki et al., 1995; Kasai et al., 1997; Taira et al.,1998; Hosaka et al., 2000; Sengupta and Rao, 2002), cell growth regulation (Ishida et al., 2002; Yang and Hecht, 2004) and microRNA stability (Yu and Hecht, 2008). Translin assembles into octamers (or decamers in *Drosophila*) *in vitro* (VanLoock et al., 2001; Sugiura et al., 2004; Gupta et al., 2008) and binds single stranded DNA and RNA sequences (Yang et al., 2003; Jacob et al., 2004; Cho et al., 2005). It also creates heteromeric complex with TRAX protein that is likewise implicated in nucleic acid binding (Vargheese et al., 2001; Finkenstadt et al., 2002; Li and Baraban, 2004, Gupta et al., 2005). Translin and TRAX are paralogous proteins, suggesting that they functionally co-evolved. Indeed, TRAX expression is under the tight control of translin and this trait is evolutionary conserved from yeast to humans (Chennathukuzhi et al., 2003; Laufman et al., 2005; Claussen et al., 2006). Furthermore, it seems likely that TRAX regulates translin affinity for either RNA or DNA (Chennathukuzhi et al. 2001; Lluis et al., 2010). Fission yeast translin and TRAX proteins share the main biochemical characteristics with translin/TRAX complexes of

higher eukaryotes (Laufman et al., 2005). Yet, the biological function of Tsn1/TRAX complex in yeasts is unknown, but the experimental evidence of Jaendling et al., (2008) suggests that it is not involved in DNA recombination.

Translin is reported to be predominantly cytoplasmic protein, however, it is known to localize to nucleus in hematopoetic cell lines and in HeLa cells in a response to DNA-damaging drugs (Aoki et al., 1995; Kasai et al., 1997). It was also found to be specifically associated with centrosomes, bipolar mitotic splindles and midbodies in mice and centrosomes in *Xenopus* oocytes (Castro et al., 2000; Ishida et al., 2002). In large scale protein localization study with fission yeasts it was found mainly in the nucleus, but also in cytoplasm (Matsuyama et al., 2006). My experiments did not validate the data form this study. First, I could not detect the GFP-tagged Tsn1 protein in cells where the *tsn1* gene was tagged with *GFP* on chromosome, suggesting that GFP tag interferes with Tsn1 expression or Tsn1 protein stability. Second, I observed the Tsn1-HA signal mainly in the cytoplasm of formaldehyde fixed *wt + pMGT* and *Δtsn1 + pMGT* cells (Fig. 3.25. and 3.26.). Nevertheless, it is still possible that a small portion of Tsn1 localizes to the cell nucleus. In fact, the Tsn1-ChIP data suggest that at least a part of Tsn1 is associated with nuclear chromatin in a way similar to Rct1 (Fig. 3.27.).

My pull down experiments revealed that Rct1 interacts with the recombinant Tsn1 protein *in vitro,* and that both PPIase and RRM domains are involved in the interaction (Fig. 3.3. and 3.5.). The Rct1 RS domain is also required for the interaction. The main role of RS domain in the interaction seems to be stabilization of PPIase and RRM domain

structure, but we also cannot exclude the direct participation of RS domain in the interaction. Similar to SR proteins, I could not reproduce the pull down interaction of translin with Rct1 employing the co-immunoprecipitation assay. Again, one possible explanation is that the *in vivo* interactions are only transient or phosphorylation-dependent and therefore under the detection limit of the co-immunoprecipitation assay. Another possibility is that Rct1 interacts only with Tsn1 monomers or homomers that are likely not abundant in the cell. Yet, this kind of interaction might be important for the assembly of functional Tsn1/TRAX heteromeric complexes. Indeed, one of the putative Rct1 target sites lies within the loop region and the proper structure of this loop was reported to play an essential function in the translin oligomerization in *Drosophila* (Gupta et al., 2008). I also tested Tsn1 for the interactions with fission yeast SR proteins and splicing related kinases. Surprisingly, the pull down experiments revealed that Tsn1 interacts with splicing factor Prp2 (Potashkin et al., 1989; Webb et al., 2005; Sridharan and Singh, 2007) and splicing related Prp4 kinase (Alahari et al., 1993; Gross et al., 1997), suggesting that Tsn1 might be, like Rct1, involved in the pre-mRNA processing.

Neither deletion nor overexpression of *tsn1* gene has any reproducible effect on the viability or cell morphology of the fission yeasts (see Fig. 3.20. and Laufman et al., 2005; Jaendling et al., 2008). Interestingly, the expression levels of Tsn1 and Rct1 mRNAs are increased during the meiotic differentiation, being highest during the first and second meiotic division (Mata et al., 2002), suggesting that the two proteins might be required for a meiosis-specific process. Accordingly, I could show that Tsn1 protein level increases during the meiosis I and II (Fig. 3.23.). Furthermore, my experiments revealed that both

overexpression and deletion of *tsn1* in diploid cells result in significantly decreased sporulation efficiency in otherwise wild type cells (Fig 3.21.). This suggests that a precise amount of Tsn1 is required for proper meiotic differentiation. By introducing the *pat1-114/pat1-114* mutation into the *Δtsn1/Δtsn1* strain the decreased sporulation efficiency of *Δtsn1/Δtsn1* strain was fully restored (Fig. 3.24.). It is therefore possible that Tsn1 is needed, directly or indirectly, to deactivate the negative regulator of meiosis Pat1 that acts at an early stage of meiosis. It was previously reported that Rct1 negatively regulates sporulation efficiency in a dosage-dependent manner (Gullerova et al., 2007), whereas I showed here that the *rct1* gene deletion is epistatic over the *tsn1* overexpression (Fig. 3.12.). It is therefore possible that Rct1 regulates the activity of Tsn1 during the meiotic differentiation. Although the precise function of Tsn1 in meiosis is unclear, the interaction of Tsn1 with Prp4 and Prp2 and the fact that Rct1 influences splicing of meiotic genes (Gullerova et al., 2007) suggest that both genes might act together to regulate meiosis-specific transcription and/or splicing.

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6. CURRICULUM VITAE

PERSONAL DATA	
• Name	**Hana**
• Surname	**Kautmanova**
• Middle name(s)	-
OTHERS:	
• Date of birth	2nd November 1983
• Place of birth	Bratislava, Slovak Republic
• Nationality	Slovak
• Languages	Slovak (native), English (fluent), Czech (fluent), Spanish (basic), German (basic)
EMPLOYMENT	
• Dates (from – to)	**1. 10. 2007- 30. 11. 2010**
• Name and type of organisation providing employment	Medical University of Vienna (at Max F. Perutz Laboratories)
• Position	PhD student researcher
EDUCATION AND TRAINING	
• Dates (from – to)	**1. 10. 2008- continue**
• Name and type of organisation providing education and training	University of Vienna
• Principal subjects/occupational skills covered	PhD training in molecular biology
• Title of qualification awarded	
• Level in national classification	
• Dates (from – to)	**2005-2007**
• Name and type of organisation providing education and training	Comenius University, Faculty of Natural Sciences, Department of Genetics
• Principal subjects/occupational skills covered	master's degree training in genetics and molecular biology

• Title of qualification awarded	awarded by master's degree in May 2007
• Level in national classification	diploma thesis A, Special genetics B, Special molecular biology A, Molecular biology and genetics of microorganisms A
• Dates (from – to)	**2002-2005**
• Name and type of organisation providing education and training	Comenius University, Faculty of Natural Sciences
• Principal subjects/occupational skills covered	Bachelor's degree training in biology with special interest in genetics and molecular biology
• Title of qualification awarded	awarded by bachelor's degree in June 2005
• Level in national classification	bachelor's thesis A, Molecular biology A, Genetics A
• Dates (from – to)	**1998-2002**
• Name and type of organisation providing education and training	Gymnasium Jura Hronca, Novohradska 1, 82100, Bratislava
• Principal subjects/occupational skills covered	high school

CONFERENCES

• Dates (from – to)	**26.10. – 31.10. 2009**
• Name of meeting	The fifth international fission yeast meeting, Pombe 2009
• Place of meeting	Tokyo, Japan
• Type of presentation	poster
• Name of presentation	The role of peptidyl prolyl isomerise Rct1 in meiotic progerssion

WORKSHOPS AND TRAININGS

• Dates (from – to)	**13.6. – 27.6. 2009**
• Name and type of organisation providing education and training	European Molecular Biology Organisation, EMBO
• Principal subjects/occupational skills covered	Practical Course *"Molecular genetics with the fission yeast Schizosaccharomyces pombe"*
• Dates (from – to)	**30.3. – 2.4. 2009**
• Name and type of	International Centre for Genetic Ingineering and

organisation providing education and training	Biotechnology, ICGEB
• Principal subjects/occupational skills covered	Theoretical Course *"RNA structure and Function"*
• Dates (from – to)	**13. 1. - 15. 1. 2009**
• Name and type of organisation providing education and training	European Molecular Biology Organisation, EMBO
• Principal subjects/occupational skills covered	EMBO workshop on *"Regulatory RNAs in Pro- & Eukaryotes"*

LABORATORY EXPERIENCES

• Dates (from – to)	**2007 - 2010**
• position	PhD student
• lab head	Dr. **Lorkovic Zdravko**
•institute/department/ place/country	Max F. Perutz Laboratories, Department of Molecular Biology and Biochemistry, *Dr. Bohr-Gasse 9, 1030 Wien, Austria*
•name of project	The role of *Schizosaccharomyces pombe* cyclophilin Rct1 in coupling splicing to RNA polymerase II transcription
• Dates (from – to)	**2004 – 2007**
• position	Diploma student
• lab head	Prof. **Tomaska Lubomir**
•institute/department/ place/country	Comenius University - Faculty of Natural Sciences, Department of Genetics, *Mlynska dolina B-1, 842 15 Bratislava, SLOVAKIA*
•name of project	Vacuolar and mitochondrial communication induced by nigericin in the yeasts *Saccharomyces cerevisiae*