

Investigating the links between the nuclear
RNA exosome and RNA export in fission
yeast

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Declaration

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 - Wild-type mCherry strains were created by Dr. Adrien Birot, Vasilieva Lab (Section 2.10)
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Abstract

Production of the correct amount of functional RNA molecules is vital for cell survival. This can be achieved at different times during the life of an RNA molecule, starting with RNA synthesis and ending at RNA degradation, whilst passing through maturation and transport inside the cell. In eukaryotes, an important player in RNA regulation is the essential multi-protein 3' to 5' RNA exosome complex. This molecular scissor monitors the correct maturation and levels of a diverse variety of protein-coding and non-coding RNA transcripts as well as plays a role in the export of RNAs from the nucleus to the cytoplasm. However, the molecular mechanism underpinning various functions of the exosome in RNA regulation are not well understood. The work presented here explores the possible links between a predicted export protein Sbp1, component of Ran GTPase complex and the nuclear RNA exosome. This study characterises Sbp1 and links Sbp1 to Dis3, the main catalytic RNase of the exosome, accentuating the relation between RNA export and decay. Secondly, this study uses the data collected from the poly(A)+ interactome to investigate possible mechanisms involved in substrate recognition by the nuclear RNA exosome. These results propose a two-step mechanism of RNA substrate recognition in which a specific ribonucleoparticle is assembled and then targeted by the nuclear exosome, which is coordinated by the essential helicase Mtl1/Mtr4.

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Abbreviations

CPF	cleavage and polyadenylation factor
CUT	cryptic unstable transcript
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
ESF	exosome specific factor
FISH	fluorescent in-situ hybridisation
GFP	green fluorescent protein
GO	gene ontology
<i>H. sapiens</i>	<i>Homo sapiens</i>
IGB	integrated genome browser
KOW	kryprides ouzounis woese domain
mRNA	messenger RNA
MS	mass spectrometry
MTREC	Mtl1-Red1 core
m3G	tri-methyl-guanosine
m7G	7-methylguanosine
nc	non-coding
NPC	nuclear pore complex
OD ₆₀₀	optical density measured at 600nm
ORF	open reading frame
Pabs	poly(A) binding proteins
PBS	phosphate-buffered saline

pre	premature
Ran	Ras-related nuclear protein
RBD	Ran-binding domain
RanBP	Ran-binding protein
RanGAP	Ran GTPase activating protein
RanGEF	Ran guanine nucleotide exchange factor
RBP	RNA-binding protein
rRNA	ribosomal RNA
RNA	ribonucleic acid
RNAP	RNA polymerase
RNA-seq	RNA sequencing
RNP	ribonucleoprotein
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>
SDS	sodium dodecyl sulfate
sm	single molecule
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
ts	temperature sensitive
TRAMP	Trf4/5, Air1/2 and Mtr4 complex
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
UTR	untranslated region
WT	wild-type
YES	yeast extract supplemented

Chapter 1

Introduction

The production of an adequate amount of functional RNA molecules is essential for cell viability. In eukaryotes, non-coding (nc) RNA and protein-coding (m) RNA species are derived from transcription of a DNA template carried out by RNA polymerases (RNAP). In eukaryotes, three RNAP isoforms exist (RNAP I, II, and III) and each RNAP transcribes a specific subset of RNA. RNAP II is involved in the transcription of all mRNA, along with the transcription of small nucleolar (sno) RNAs and all small nuclear (sn) RNAs (U1, U2, U4, U5) except U6. Transcription by RNAP II also produces unstable ncRNA species, including cryptic unstable transcripts (CUTs), a subset of ncRNAs that are formed due to ‘cryptic’ transcription and that are identified once RNA surveillance machineries are compromised (Neil et al. 2009, Davis & Ares 2006, Vasiljeva et al. 2008). Lastly, RNAP I is responsible for the transcription of most ribosomal (r) RNAs (5.8S, 18S and 25S) and RNAP III synthesises the remaining 5S rRNA, along with transfer (t) RNAs and U6 snRNA. After RNA synthesis is initiated, RNA undergo processing events, some of which occur during transcription and some, such as 3’ end trimming by the exosome, occur after transcription.

Multiple RNA-binding proteins are involved in regulation of each step of RNA biogenesis, including RNA production, processing, transport, and decay. Firstly, the initial premature (pre) RNA transcript is synthesised by the respective RNAP. Immediately after transcription is initiated, this pre-RNA is rendered available for the processing that is required to ensure production of a functional mature RNA

molecule. Processing includes several steps that can occur during transcription (co-transcriptionally) or after transcription (post-transcriptionally). Transport of RNA adds an additional level of regulation and many different RNA subtypes require transport through channels embedded in the nuclear envelope (nuclear pore complexes, NPC). The NPC can be viewed a portal into the cytoplasm and if an RNA is required in the cytoplasm it will traverse across the NPC, either by binding the NPC directly or with the help of export-promoting RNA-binding proteins. Unsurprisingly, mRNA require transport across the NPC to the cytoplasm where they get translated into proteins and some ncRNAs also undergo NPC transport to the cytoplasm. A further level of cellular RNA regulation is that of RNA degradation. RNA can be exonucleolitically degraded in both 5' to 3' and 3' to 5' directions and the RNA exosome plays a role as the major 3' to 5' RNA degrading machinery and as one of the main focuses of this study. Specifically, this thesis focuses on aspects of RNA biogenesis that link RNA transport and the RNA exosome.

1.1 RNA processing

Processing of pre-RNA can occur co-transcriptionally or post-transcriptionally and this processing is critical for the formation of functional and mature RNA molecules. In eukaryotes, RNA processing is mainly achieved by big multi-protein complexes.

1.1.1 mRNA processing

Processing of mRNA occurs co-transcriptionally and includes capping, splicing, and cleavage and polyadenylation, where these processes are linked to specific stages of transcription, as reviewed in Bentley (2014). Capping takes place early on during transcription and involves the addition of a mono-methyl cap (7-methylguanosine, m7G) to the 5' end of RNA as the transcript becomes exposed from RNAP II. This cap is essential for protecting RNA from degradation by nuclear 5' to 3' exonucleases and is critical for the later co-transcriptional processing events, RNA

stability, export, and translation (Bentley 2014). Similarly, during transcription elongation, step-wise recruitment of the large multi-protein spliceosome complex and the subsequent splicing of introns occur. Lastly, at the end of transcription, cleavage of mRNA and polyadenylation (addition of a poly(A) tail) is triggered once RNAP II reaches the area corresponding to the polyadenylation (pA) site (Bentley 2014). Cleavage and polyadenylation are coupled by the cleavage and polyadenylation factor (CPF) complex, a multi-protein complex consisting of catalytic modules (Bentley 2014). The polyadenylation module of the CPF recognises the pA site and polyadenylates the 3' end of the mRNA after cleavage downstream of the pA site by an additional module with nuclease activity. Like the recruitment of processing factors, proteins required for mRNA export are also recruited co-transcriptionally together with other proteins to form a functional export-competent mRNA collectively termed a messenger ribonucleoprotein (RNP) particle.

1.1.2 ncRNA processing

Many ncRNA are subjected to similar processing events to mRNAs and these processing events can occur co- or post-transcriptionally. The processing of many pre-ncRNAs can also include 5' capping and 3' polyadenylation (Cai et al. 2004, Mouaikel et al. 2002) and some RNAP II transcribed ncRNAs have also been observed to undergo splicing in higher eukaryotes (Krchňáková et al. 2019, Quinn & Chang 2017).

Mammalian nuclear RNAs (sn/snoRNAs) are well characterised to shuttle between the nucleus and cytoplasm in mammals as a prerequisite for their role in the nucleus for the splicing of pre-mRNA (Fischer & Luhrmann 1990, Zieve et al. 1988). Therefore, processing of this nuclear subtype of RNA can occur in both the nucleus and the cytoplasm of mammalian cells. First, sn/snoRNAs are transcribed by RNAP II in the nucleus, receiving a m7G cap co-transcriptionally and then in the cytoplasm this cap can be further methylated to convert the m7G cap to a 2,2,7-trimethylguanosine or m2,2,7G cap (Mouaikel et al. 2002, Will &

Lührmann 2001). Cytoplasmic pre-snRNA maturation also includes the binding of snRNAs to the ‘Sm’ ring, a process that is vital for the formation of the spliceosome and for further processing events (Will & Lührmann 2001). In the nucleus, the 3’ end of pre-snRNAs can be trimmed by the exonucleolytic nuclear exosome complex (Allmang et al. 1999, Coy et al. 2013).

Differently to RNAP II-transcribed snRNAs, RNAP III-transcribed U6 snRNA receives a γ -monomethylphosphate cap and this modification also occurs in the processing of tRNAs. tRNAs, rRNAs, and snRNAs are also heavily modified within their bodies and many of these modifications are important for later processing events (Roundtree et al. 2015). Examples include base isomerisation of tRNAs, base methylations of rRNAs, and pseudouridination (isomerisation of uridine bases) of snRNAs, as reviewed in Roundtree et al. (2015). Lastly, post-transcriptional maturation from pre-rRNA, tRNA, and telomerase RNA to mature transcripts also includes 3’ end trimming by the exosome complex (Allmang et al. 1999, Mitchell et al. 1997, Schneider et al. 2012, Coy et al. 2013).

1.2 Nuclear RNA exosome structure

The multi-protein nuclear exosome complex is composed of six PH-ring proteins (Figure 1.1, blue) organised around a central channel and three RNA-binding proteins capping the channel (Figure 1.1, green) (Kowalinski et al. 2016, Liu et al. 2006, Mitchell et al. 1997, Wasmuth et al. 2014). These nine proteins form the catalytically inactive core of the nuclear RNA exosome (EXO9) (Figure 1.1). The catalytic activity of the exosome is provided by the association of EXO9 with two nucleases (Dziembowski et al. 2007, Makino et al. 2013). The exonuclease Rrp6 binds the cap of EXO9 (Figure 1.1, red), whereas the exo- and endoribonuclease Dis3 binds at the base of EXO9 (Figure 1.1, purple). Rrp6 also requires proteins Rrp47 and Mpp6 for its full activity (Figure 1.1, grey) (Wasmuth et al. 2017). The channel through the core of the exosome can accommodate single stranded RNA and RNA is fed through the channel to reach Rrp6 and Dis3.

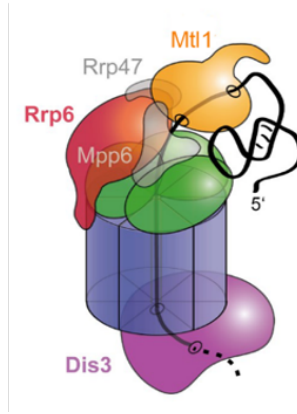


Figure 1.1: **Schematic representation of the nuclear RNA exosome.** The Nuclear RNA exosome consists of 11 proteins formed of a nine-protein core that associates with 2 nucleases: the exonuclease Rrp6 (red) and the exo- and endoribonuclease Dis3 (purple). RNA is represented by a black line. Figure kindly provided by Lidia Vasilieva from Kilchert et al. (BioRxiv, 2019, accepted to Genome Research 2020).

1.3 Nuclear RNA exosome functions

In addition to its role in the 3' end trimming of ncRNAs discussed above, the nuclear RNA exosome is important for the complete degradation of unstable ncRNA molecules, including the removal of the RNAP II-transcribed CUTs (Zhou et al. 2015, Wyers et al. 2005, Vasiljeva et al. 2008). The exosome also participates in RNA quality control by degrading aberrant transcripts such as those that failed to undergo processing. For example, mRNAs that failed to undergo proper splicing or 3' end processing by the CPF are all targeted to the exosome (Bitton et al. 2015, Bousquet-Antonelli et al. 2000, Hilleren et al. 2001).

Additionally, the RNA exosome appears to have a role in the regulation of gene expression through the maintenance of proper levels of specific mRNA transcripts (Harigaya et al. 2006, Lloret-Llinares et al. 2018, Mistry et al. 2012). A well characterised and striking example of this is the role of the exosome in the regulation of transcripts involved in meiosis, cell cycle, and biosynthetic pathways (Harigaya et al. 2006, Kilchert et al. 2015, McPheeters et al. 2009, Sugiyama & Sugiyama 2011, St-Andre et al. 2010, Shichino et al. 2020, Yamanaka et al. 2010). In *Schizosaccharomyces pombe* (*S. pombe*, fission yeast),

this elimination of meiosis-specific transcripts is carried out by the conserved YTH domain protein Mmi1, which is co-transcriptionally recruited to a specific consensus sequence UUA AAC to mediate the degradation of these transcripts by the exosome (Yamashita, Shichino, Tanaka, Hiriart, Touat-Todeschini, Vavasseur, Ding, Hiraoka, Verdel & Yamamoto 2012, Harigaya et al. 2006, Hiriart et al. 2012, Kilchert et al. 2015). Recently, it has also been proposed that the nuclear exosome is important in the regulation of cell cycle progression (Snee et al. 2016) and in the expression of developmental programs (Burns et al. 2018, Boczonadi et al. 2014).

1.3.1 Compromised exosome function leads to multiple defects in RNA biogenesis

Highlighting the essential role of the exosome in RNA regulation, exosome mutants exhibit a broad range of phenotypes which extend beyond the functions of the exosome directly associated with its nucleolytic RNA degradation described in Section 1.3.

Notably, depletion of Dis3 and Rrp6 has been shown to be linked to a build-up of mRNAs and ncRNAs harbouring 3' extended ends and to defective RNAP II termination at the end of transcription (Davidson et al. 2019, Lemay et al. 2014, Shah et al. 2014, Torchet et al. 2002, Van-Hoof et al. 2000). For instance, Dis3 depletion was shown to enhance the association of RNAP II with chromatin downstream of the pA site, suggesting that the exosome can play a role in promoting the transcription termination of paused or backtracked RNAP II and subsequent degradation (Lemay et al. 2014).

Interestingly, Rrp6 exosome mutants also accumulate transcripts that have longer poly(A) tails (hyperadenylated transcripts) (Bresson & Conrad 2013, Hilleren & Parker 2001, Hilleren et al. 2001, Yamanaka et al. 2010). Hyperadenylation has been shown to be lost upon mutation or depletion of the fission yeast CPF associated poly(A) polymerase Pla1 (PAPOLA in humans), indicating that the majority of this hyperadenylation is mediated by the CPF component Pla1 (Yamanaka

et al. 2010). Additionally, poly(A) binding proteins (Pabs) have been shown to promote hyperadenylation and point mutations in Pabs, including of mammalian PABPN1 and fission yeast Pab2, suggesting that Pabs are necessary to couple hyperadenylation with the decay of a subset of nuclear RNAs (Bresson & Conrad 2013). Hyperadenylation has also been identified upon deletion of Pab2 in fission yeast (Perreault et al. 2007) along with the identification of an accumulation of several snoRNAs harbouring extended 3' tails, again coupling hyperadenylation with decay in fission yeast (Lemay et al. 2010, Perreault et al. 2007).

Additionally, phenotypes associated with defects in pre-mRNA splicing (Kilchert et al. 2015), 3' end processing, and transcription termination (Fox et al. 2015, Lemay et al. 2014, Shah et al. 2014) have also been observed when exosome function is compromised. Further, dramatic accumulation of poly(A)+ RNA in the nucleus has also been observed in exosome mutants, suggesting that RNA export is also compromised when the exosome is inactivated (Assenholt et al. 2008, Carneiro et al. 2007, Paul & Montpetit 2016, Thomsen et al. 2003). Although nuclear retention has been demonstrated for individual mRNAs by single molecule (sm) fluorescent in-situ hybridisation (FISH), at present the complete list of RNAs that are retained in the nucleus is lacking. Currently, the molecular mechanisms underpinning these phenotypes and the role of the exosome in contributing to these effects are not clear. It is also unclear which of these effects are a direct result of impaired exosome function and which are an indirect effect resulting from de-regulated exosome activity.

This accumulation of RNA in the nucleus can be detrimental to the cell. RNA accumulation has been linked to RNA-DNA hybrids arising from excess nuclear RNA (Whaba et al. 2013) and R loop formation (Aguilera & Garcia-Muse 2012, Sollier & Cimprich 2015), both of which are common causes of genome instability. Loss of heterochromatic silencing (Bronner et al. 2017) and deregulation of polycomb targets (Garland et al. 2019) has also been proposed to be linked to the accumulation of exosome targets in the nucleus. Accumulation of RNA may result in transcriptional defects, for instance accumulated RNA has been suggested to compete for RNA-binding proteins and so to titrate RNA-binding proteins away

from processed RNA (Biro et al. unpublished manuscript from the Vasilieva lab, 2020). For instance, CPF association with poly(A)+ RNA was shown to be increased in exosome mutants, suggesting that nuclear accumulation could result in the prevention of CPF recycling in exosome mutants (Biro et al. unpublished manuscript from the Vasilieva lab, 2020).

Considering the effects of compromising the exosome on the transcript level, it is unsurprising that improper exosome function has been associated with disease pathology in humans. Dereglulation of the exosome has been linked to several neurological diseases, including spinal muscular atrophy (Boczonadi et al. 2014) and pontocerebellar hypoplasia (Wan et al. 2012, Biancheri et al. 2013, Rudnik-Schöneborn et al. 2013).

1.4 Regulation of the nuclear RNA exosome

It has been demonstrated that the exosome on its own is not a specific or an efficient enzyme (Liu et al. 2006, Wasmuth & Lima 2012). Despite this, the exosome can be efficiently targeted to specific RNAs *in vivo* to degrade a large repertoire of RNA. This raises the question of which proteins are important for exosome regulation.

Although a number of RNA binding factors have been linked to the exosome, the mechanisms by which they regulate exosome are not understood. It is becoming ever more evident that RNA-unwinding capabilities are central to exosome control. In *Saccharomyces cerevisiae* (*S. cerevisiae*, budding yeast) the two helicases nuclear Mtr4 and cytoplasmic Ski2 have been shown to be important for RNA degradation by the exosome in the nucleus and cytoplasm respectively (Hardwick & Luisi 2013). Mtr4 (Figure 1.5A) is known to associate with proteins that bind to RNA and to the exosome itself (Weick et al. 2018, Lubas et al. 2011, Lingaraju et al. 2019). For example, Mtr4 has been shown to associate with the RNA-binding zinc finger proteins Air1/2 (Air1 is only present in *S. pombe*), along with a non-canonical poly(A) polymerase, to form the TRAMP (Trf4/5-Air1/2-Mtr4) complex (Bühler et al. 2008, LaCava et al. 2005, Vanáčová et al. 2005). The poly(A) polymerase

activity of TRAMP is utilised to add a short poly(A) tail to a select number of nc transcripts. As a part of the TRAMP complex, Mtr4 is required for degradation (CUTs) or trimming (sn/snoRNAs, tRNAs, rRNAs) by the exosome (Jia et al. 2011).

A Mtr4-containing TRAMP complex is conserved in fission yeast (Bühler et al. 2007, Keller et al. 2010) where it is mainly limited to rRNA (Bühler et al. 2007, Win et al. 2006). Fission yeast Mtr4 also shares its role with another helicase, the Mtr4 homologue Mtl1 (Mtr4-like protein 1) (Figure 1.5A). Like Mtr4, Mtl1 has been shown to be able to associate with proteins that bind to RNA as well as to proteins that bind to the exosome itself, including the zinc finger protein Red1 (proposed MTREC complex, Mtl1-Red1 core) which assists the nuclear exosome in the turnover of CUTs and aberrant mRNAs (Lee et al. 2013, Zhou et al. 2015), Mmi1, Iss10, nuclear poly(A) binding proteins Pab2, and proteins that bind to the 5' cap of RNA (Cap-binding proteins, Cbc1, Cbc2 and Ars2) (Egan et al. 2014, Zhou et al. 2015).

Mtr4 and Mtl1 are components of two separate complexes in fission yeast, TRAMP and MTREC respectively, whereas Mtr4 is only present in TRAMP in budding yeast, reviewed in Kilchert et al. (2016). Similarly, although Mtl1 is absent in humans, human Mtr4 (hMTR4) is a component of two distinct complexes that associate with the exosome (Lubas et al. 2011, 2015, Meola et al. 2016). Therefore, Mtl1/Mtr4 is implicated in multiple aspects of exosome targeting to RNAs in humans and fission yeast and thus has a less prominent role than in *S. cerevisiae*.

1.5 Nuclear export

Presence of the nuclear envelope results in two distinct cellular compartments in a cell, the nucleus and the cytoplasm. Due to this separation, many RNA species require export through the NPCs embedded in the nuclear envelope. Many RNAs are transported across the nuclear envelope after transcription and any processing events that are required for the specific subtype (as discussed previously).

Nucleocytoplasmic transport of RNA is carried out by the association of the RNA ‘cargo’ with specific proteins that facilitate the transport of RNA through the NPC (Kohler & Hurt 2007) (Figure 1.2). Nuclear transport receptors are an example of these specific proteins that associate with the RNA (Figure 1.2). Although some RNA species (e.g. tRNAs) can be exported by binding directly to nuclear transport receptors (Figure 1.2), other larger RNAs such as mRNAs form an RNP particle that then associates with other proteins called adaptor proteins that recruit the nuclear transport receptors (Kohler & Hurt 2007) (Figure 1.2). Despite the notion of nucleocytoplasmic transport being similar, the precise mechanism behind the transport differs for different RNA subtypes.

1.5.1 Nuclear export of mRNA

Export factors are co-transcriptionally recruited to the nascent RNA and therefore mRNA export is coupled to transcription. Studies from mammals show that the main export receptor required for mRNA export is the TAP-p15 heterodimer (otherwise known as NXF1-NXT1) and the homologue of this complex Mex67-Mtr2 functions as the most utilised mRNA export receptor in *S. pombe* (Santos-Rosa et al. 1998, Segref et al. 1997). Numerous additional export-related proteins associate with the RNP and are often utilised to promote export. For instance, Mlo3, the fission yeast homologue of mammalian export adaptor ALYREF, has been suggested to be an adaptor for Mex67-Mtr2 (Gopal et al. 2005). These adaptor proteins can recruit the export receptors and promote the export of the RNA through the NPC.

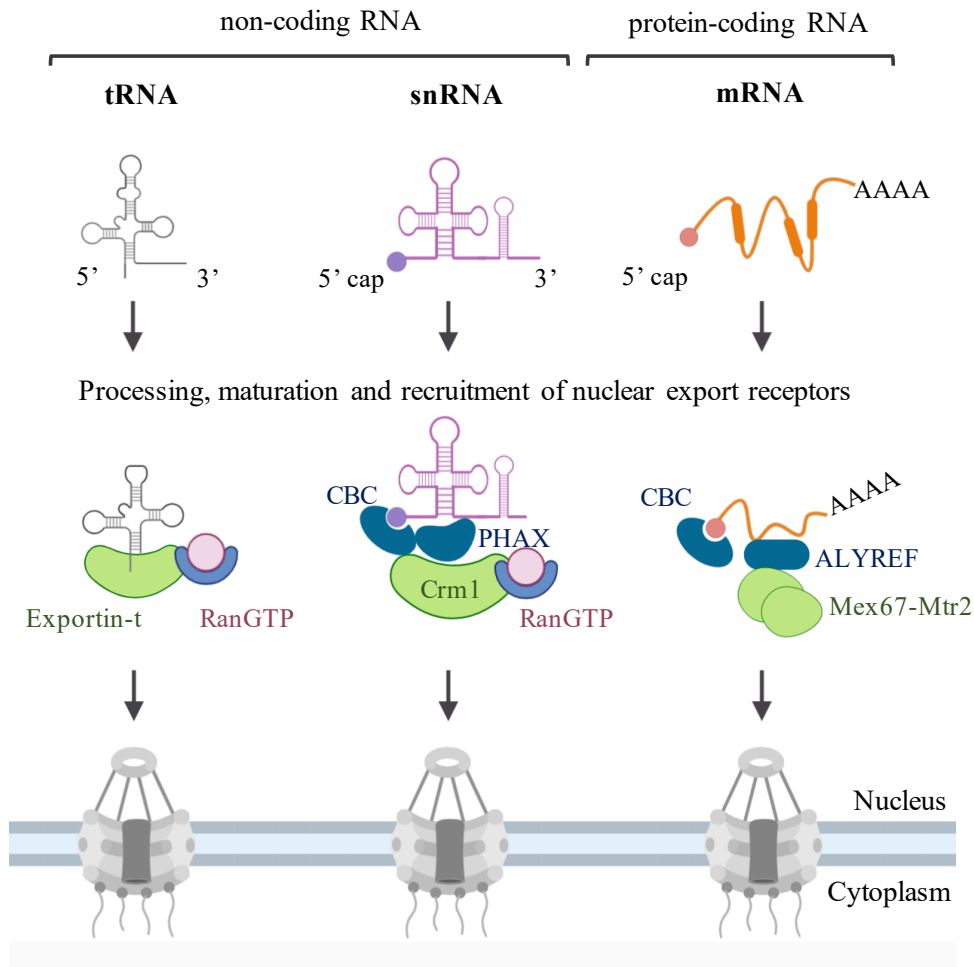


Figure 1.2: Schematic representation of different mechanisms of RNA export. Examples of RNA export across nuclear pore complexes embedded in the nuclear envelope. Examples shown for non-coding RNA (transfer RNA tRNA, small nuclear RNA snRNA) and protein coding RNA (mRNA). After transcription of a primary RNA, RNA can undergo processing, maturation, and the recruitment of proteins to form an export-competent RNA. Primary RNA and export-competent RNA are shown for each subtype. Proteins involved with export which can associate with RNA are demonstrated for each RNA subtype: Nuclear export receptors (green), RanGTP (pink), and adaptor proteins (blue). Examples of nuclear export receptors are shown on the diagram in green: Exportin-t, Crm1, and Mex67-Mtr2/TAP, and examples of adaptor proteins are shown in blue (cap-binding complex, CBC and ALYREF). 5' cap and 3' poly(A) tail are demonstrated where appropriate. Arrows indicate the direction of movement from the nucleus to the cytoplasm. Schematic was created with Biorender and based on Kohler & Hurt (2007).

1.5.2 Nuclear export of ncRNA

As shown in Figure 1.2, many ncRNAs (e.g. snRNAs, tRNAs) also require nucleocytoplasmic transport. Export of snRNAs is required for maturation of the pre-snRNA (as discussed in Section 1.1.2), whereas transport of tRNAs into the cytoplasm is necessary for pre-tRNA maturation and for later roles in ribosomal translation (Kohler & Hurt 2007) (Figure 1.2). Additionally, pre-rRNAs are transcribed in the nucleolus and transported to the cytoplasm to synthesise ribosomal proteins (Kohler & Hurt 2007).

A classical ncRNA transport route, utilised for the export of tRNA, snRNA, telomerase RNA, and rRNA depends on the karyopherin family of export receptors (Kohler & Hurt 2007). Karyopherins can be subdivided into those that promote export (exportins) or promote import (importins) (Kohler & Hurt 2007) (Figure 1.2). Many studies in mammals have identified a variety of exportins that are important for and involved in RNA transport, including Exportin-t and Crm1 (otherwise called Exportin-1), which are tRNA and snRNA/rRNA export receptors respectively (Kohler & Hurt 2007) (Figure 1.2).

The ability of an exportin to bind to RNA is dependent on the ‘GTP state’ of Ran (Ras-related nuclear protein) in the Ran cycle, a well characterised cycle in mammals. This Ran-dependence is unique to ncRNA export and results from the cooperation between multiple proteins (Figure 1.3):

1. Ran: a nuclear and cytoplasmic localised protein with enzymatic activity (GTP hydrolysis) that provides the energy for the transport of cargo through the NPC once activated. Exists in Ran-GTP or Ran-GDP forms (Bischoff & Ponstingl 1991*b,a*).
2. RCC1: RanGEF (Ran guanine nucleotide exchange factor), a nuclear localised protein involved in the displacement of the GDP of Ran to GTP (Bischoff & Ponstingl 1991*b,a*).
3. RanGAP1: RanGAP (Ran GTPase activating protein), a cytoplasmic

localised protein involved in the activation of the intrinsic GTPase activity of Ran for nucleocytoplasmic transport (Bischoff et al. 1994, 1995).

Additionally, Ran-binding proteins (RanBPs) are small nuclear and cytoplasmic proteins that act as co-activators for Ran, binding to Ran in its GTP-associated form and enhancing the activation of GTP hydrolysis by the RanGAP. RanBPs discovered in mammals include RanBP1 and RanBP2 (Bischoff et al. 1995, Yokoyama et al. 1995). RanBP1 has been identified as important for dissociating nuclear export complexes from the NPC in RNA transport (Kehlenback et al. 1999, Kohler & Hurt 2007), whereas RanBP2 has been identified to be a larger protein that constitutes the NPC (Yokoyama et al. 1995, Wilken et al. 1995). For reasons that will become evident later, RanBP2 will not be explored. RanBPs are often enriched in the cytoplasm of cells and can participate in nucleocytoplasmic shuttling alongside Ran, therefore playing a role in RNA export (Cavazza & Vernos 2016).

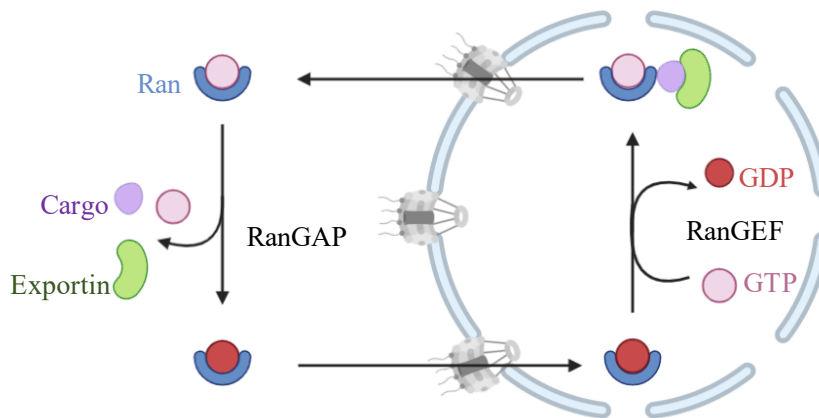


Figure 1.3: **Schematic representation of the role of the Ran cycle in nucleocytoplasmic export.** RanGEF = Ran guanine nucleotide exchange factor, RanGAP = Ran GTPase activating protein. Arrows indicate the direction of movement. Schematic was created with Biorender.

Although similar in mechanism to exportin-mediated export, export of snRNAs is further complicated by the requirement of additional proteins not required for the export of other ncRNAs (like mRNA export). Similar to export of other ncRNAs, snRNA export also depends on the karyopherin family of export receptors (Exportin-1/Crm1) and on the Ran cycle, however further steps are required to enable both the association of the export receptor and RanGTP with the cargo

prior to export and the release of cargo post export, as reviewed in Kohler & Hurt (2007). Crm1 association with snRNA requires the cap-binding complex (CBC) bound to the 5' end of the transcript and the phosphorylated adaptor for RNA export (PHAX) (Kohler & Hurt 2007). Phosphorylation of PHAX is necessary for Crm1/Exportin-1 and Ran recruitment to the CBC-bound export-competent snRNA, whereas de-phosphorylation and RanGTP hydrolysis is necessary for the release of snRNA at the cytoplasmic face (Ohno et al. 2000, Segref et al. 2001) (Figure 1.3).

Many components of the export machinery identified to be important for RNA export in mammals are present in fission yeast, however little is known about the role that they play in export. Although the exportin Crm1 has been shown to be conserved (Carobbio et al. 2001, Mercier & Labbe 2009, Neville & Rosbash 1999, Kudo et al. 1999), it is less clear whether the role as the main ncRNA receptor is the same as that suggested by mammalian studies (Kohler & Hurt 2007). Members of the Ran cycle are conserved in fission yeast and Ran cycle orthologues include: the Ran GTPase Spi1, the RanGEF Pim1, the RanGAP Rna1, and the RanBP Sbp1 (Matsumoto & Beach 1991, Sazer & Nurse 1994, Melchoir et al. 1993, He et al. 1998). Lastly, nucleocytoplasmic shuttling of snRNAs has been suggested in budding yeast (Becker et al. 2019) but has not yet been discovered for fission yeast and again it is uncertain whether this mechanism is conserved. Together this demonstrates that in comparison to mammalian studies, the requirement of components implicated in nucleocytoplasmic transport is poorly understood.

1.6 Hyperadenylation in export and exosome mutants

Nuclear accumulation of hyperadenylated transcripts observed in exosome mutants (discussed in Section 1.3.1) has also been observed in mutants of proteins required for nucleocytoplasmic export (e.g. export receptors and export adaptors) (Jensen et al. 2001, Paul & Montpetit 2016). This leads to the speculation as to whether these

phenotypes are related or instead are an indirect result of compromising both export and exosome pathways. The nuclear retention of hyperadenylated transcripts in exosome mutants might sequester factors required for export on the nuclear-retained RNA, as suggested in (Biro et al. unpublished manuscript from the Vasilieva lab, 2020, Wang et al. 2019, Fan et al. 2017, Silla et al. 2018).

1.7 ‘RNA-interactome’ capture

As discussed above, multiple phenotypes associated with RNA deregulation have been linked to defective function of the exosome, including defects in 3’ end processing, splicing, and defects in RNA export. However, the mechanisms responsible for manifesting these phenotypes, as well as which effects are a direct result of the exosome in the affected processes is not clear. Previous studies in our lab employed a system-wide approach with the aim of identifying differences in poly(A)+ RNA levels and differences in proteins associated with poly(A)+ RNA in exosome mutants compared to wild-type (WT) (Kilchert et al. BioRxiv, 2019, accepted to Genome Research 2020).

To achieve this aim, our lab adapted a poly(A)+ interactome capture method as described in Castello et al. (2013) that was previously developed to characterise proteins that associate with poly(A)+ RNA in mammalian cells. In this approach, ultraviolet (UV) light was used to cross-link RNA-binding proteins to polyadenylated RNAs, followed by the pull down of these proteins using oligo dT beads and their identification using mass spec (Castello et al. 2013) (Figure 1.4). The poly(A)+ transcriptomes and proteomes were compared between WT control and three different exosome mutants: *dis3-54*, a Pro-Leu substitution in the ribonuclease domain of Dis3 that results in a reduction in exonucleolytic activity (Murakami et al. 2007); *rrp6Δ*, deletion of the non-essential Rrp6 exonuclease (Vasilieva Lab); and *mtl1-1*, a mutant of Mtl1 with six different amino acid substitutions in the area adjacent to the arch domain of Mtl1 (Figure 1.5A) (Lee et al. 2013).

The data produced from this method were analysed in order to identify any

connection between the exosome and other RNA-binding proteins involved in RNA processing. Further, the data were analysed to assess the RNA-binding behaviour of exosome-associated factors and to discover any new factors that might be important for the regulation of the exosome in fission yeast (Kilchert et al. BioRxiv, 2019, accepted to Genome Research 2020, Birot et al. unpublished manuscript from the Vasilieva lab, 2020).

The analysis of data produced via this method demonstrated the importance of the helicase Mtl1 in exosome function, as shown by alterations in protein binding to poly(A)+ in the *mtl1-1* interactome. For instance, many exosome subunits (Rrp4, Rrp6, and Rrp40) and exosome-associated RNA binding proteins (Mpp6) were shown to be reduced on poly(A)+ RNA in the *mtl1-1* interactome (Figure 1.5B) (Kilchert et al. BioRxiv, 2019, accepted to Genome Research 2020). Despite this reduction in observed exosome components (Figure 1.5B), characterised proteins that have been implicated in exosome regulation such as CBC proteins (Cbc1, Cbc2 and Ars2) were shown to be enriched on RNA in *mtl1-1* (Figure 1.5B). Further, the YTH-domain containing protein Mmi1, a protein that has been shown to be important in exosome regulation, remains associated with RNA in *mtl1-1* (Birot et al. unpublished manuscript from the Vasilieva lab, 2020). Together these results demonstrate that the exosome recruitment to poly(A)+ RNA is reduced in the *mtl1-1* mutant, despite binding of known proteins related to exosome function not being reduced. These observations formed the basis of a two-step mechanism of exosome recruitment in which co-factors recognise target RNA and then the exosome is recruited to RNA (Birot et al. unpublished manuscript from the Vasilieva lab, 2020).

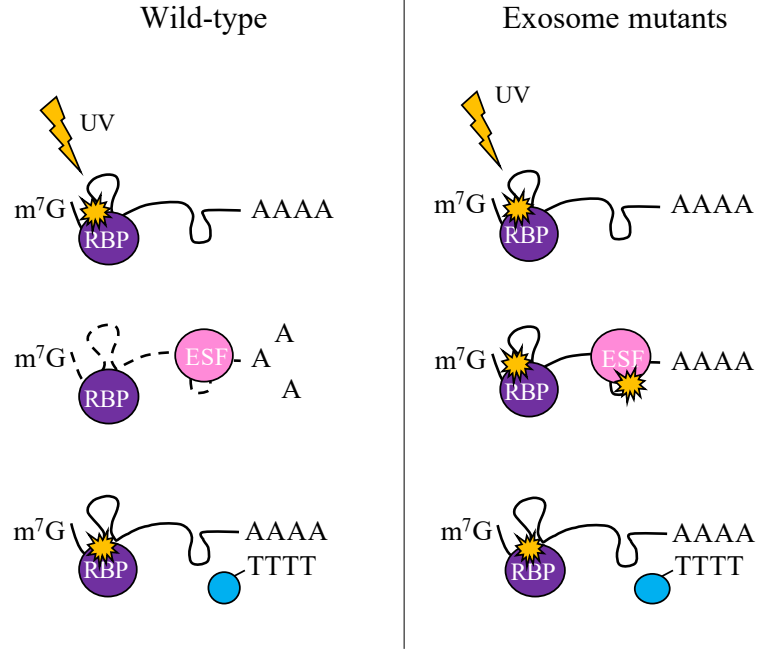


Figure 1.4: **Schematic representation of the RNA-interactome poly(A)+ RNA pull down method.** Wild-type or exosome mutant (*dis3-54*, *mtl1-1*, *rrp6Δ*) cells were grown in 4-thiouridine and subjected to ultraviolet (UV) light to cross-link and capture protein-RNA interactions. Oligo dT beads (represented by TTTT) were used to pull down intact polyadenylated RNA (represented by AAAA) and mass spectrometry and RNA sequencing was carried out on the pulled down protein-RNA. RBP = RNA-binding proteins, ESF = Exosome specific proteins. Schematic adapted from Birot et al. (unpublished manuscript from the Vasilieva lab, 2020).

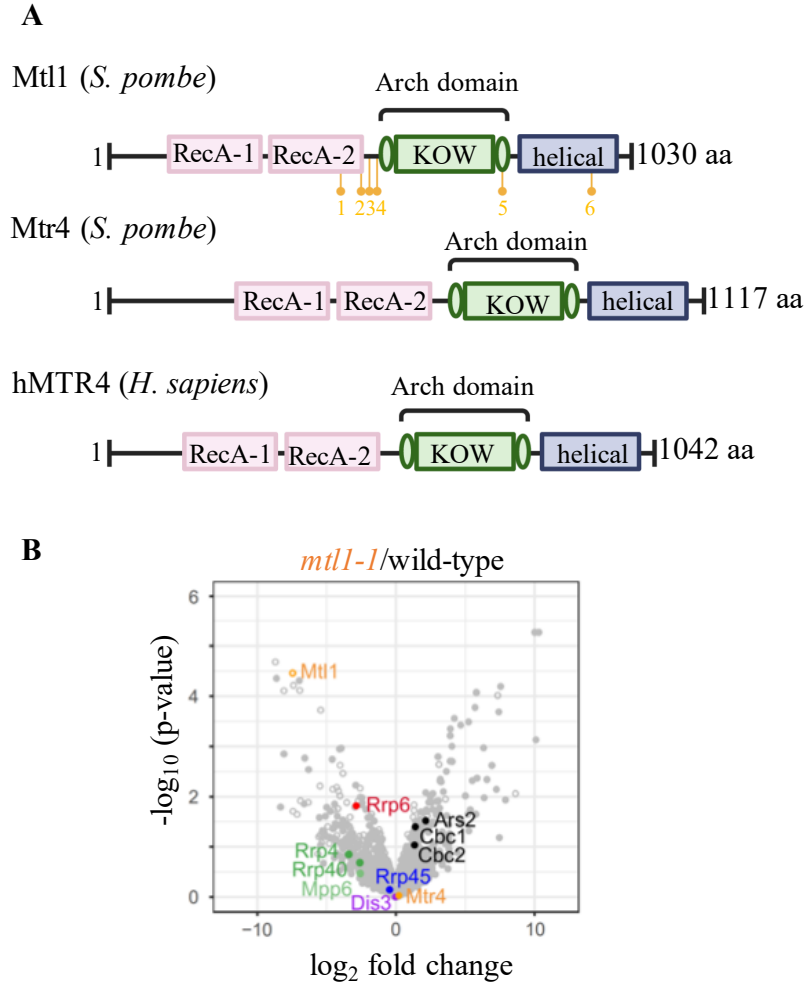


Figure 1.5: **Association of the exosome with poly(A)⁺ RNA is compromised in the *mtl1-1* mutant.** **A)** Schematic representation of Mtl1/Mtr4 helicases: *S. pombe* Mtl1, *S. pombe* Mtr4 and *H. sapiens* hMTR4 (human MTR4). Locations of the mutations in *S. pombe mtl1-1* surrounding the arch domain are annotated in yellow (I522M (1), L543P (2), Y551H (3), L557P (4), D793G (5), A998V (6)). All helicases demonstrated here have two RecA domains separated by a short flexible linker (RecA1/2, pink), KOW = kryprides ouzounis woese domain (green) and a helical domain (blue). Schematic adapted from Birot et al. (unpublished manuscript from the Vasilieva lab, 2020). **B)** Volcano plot from of comparative poly(A)⁺ RNA pull down method for *mtl1-1*. The y axis demonstrates the p-values (-log₁₀, t-test) for the comparison between of RNA interactome capture/whole cell extract ratios of poly(A)⁺ pulled down from *mtl1-1* compared to wild-type interactomes. The x axis demonstrates the -log₂ fold-change of RNA interactome capture/whole cell extract ratios in *mtl1-1* compared to wild-type interactomes. Individual proteins identified are annotated by a circle: exosome subunits (Dis3, Rrp6, Rrp4, Rrp40, Rrp45), exosome-associated RNA binding proteins (Mpp6, helicases Mtl1/Mtr4) and cap binding proteins (Cbc1, Cbc2, Ars2). Figure taken from Kilchert et al. (BioRxiv, 2019, accepted to Genome Research 2020).

1.8 Candidate protein Sbp1

To understand possible reasons for the global retention of polyadenylated RNA in the exosome mutants (discussed in Section 1.3.1), the list of RNA-binding proteins for which the association with poly(A)+ RNA was affected in the mutants was analysed to identify whether any of these proteins were predicted to also be involved in RNA export. To do this, the list of proteins was cross referenced with the list of proteins annotated by Pombase with the gene ontology (GO) ‘RNA export from nucleus’ (GO:0006405) to assess whether the occupancy of any of these RNA-binding proteins was altered in the exosome mutants. I hypothesised that the altered association of proteins involved in export could be a reason for the nuclear retention observed in exosome mutants. Previous studies have demonstrated that mutants in RNA-export pathways have also been shown to exhibit this global retention (Hilleren et al. 2001, Jensen et al. 2001, Hilleren & Parker 2001).

Interestingly, Sbp1 was identified as a protein that was both annotated by Pombase with the GO ‘RNA export from nucleus’ and as a protein for which occupancy was shown to be depleted on RNA, and this reduction of Sbp1 on poly(A)+ RNA was identified to be specific to the *dis3-54* mutant (Figure 1.6).

Sbp1 was identified to interact with human Ran GTPase via a yeast two-hybrid screen (He et al. 1998). The sequence similarity of Sbp1 to known RanBPs (Figure 1.6B) suggested that Sbp1 was a RanBP. This was confirmed by a binding assay that determined that Sbp1 could indeed bind to Ran GTPase, specifically in its GTP-associated form, and could facilitate the GTP hydrolysis cycle and the exchange of GDP/GTP (He et al. 1998). Additionally, Sbp1 was shown to physically bind the fission yeast RanGTPase Spi1 *in vitro* (He et al. 1998). Further, expression of mammalian RanBP RanBP1 was shown to rescue a mutant of Sbp1, which was otherwise inviable (Novoa et al. 1999). This rescue phenotype was also observed when the isolated Ran-binding domain of RanBP1 and Sbp1 was expressed in the Sbp1 mutant (Novoa et al. 1999). Although these data suggest that the function of Sbp1 as a RanBP is conserved from mammals to yeast, the role of Sbp1 in RNA export has never been directly confirmed and the underpinning mechanism is

unknown. To address this, I aim to characterise the interacting partners of Sbp1 in fission yeast by purifying Sbp1 native complexes from yeast and testing its role in export.

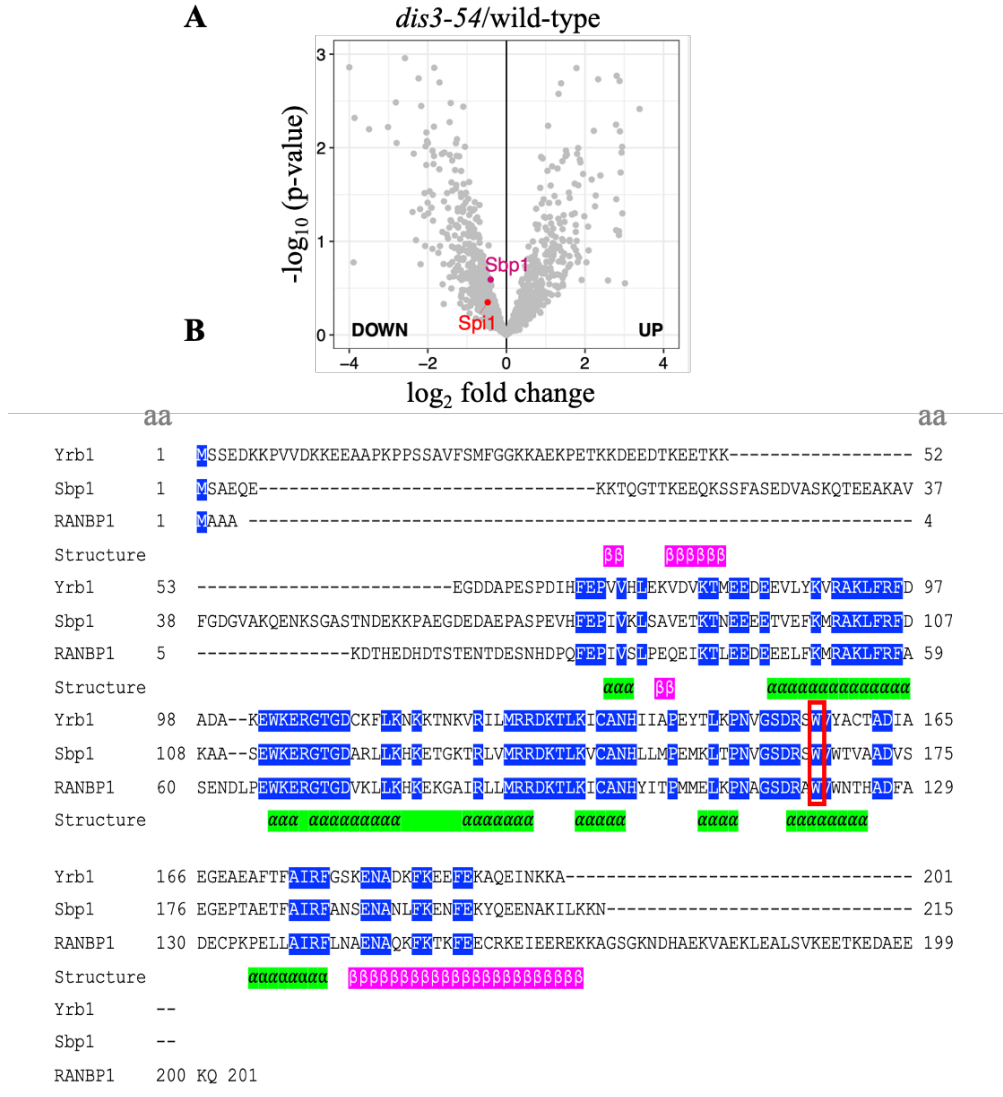


Figure 1.6: **RNA-interactome protein Sbp1.** **A)** Volcano plot of comparative poly(A)+ RNA pull down method for *dis3-54*. The y axis demonstrates the p-values ($-\log_{10}$, t-test) for the comparison between of RNA interactome capture/whole cell extract ratios of poly(A)+ pulled down from *dis3-54* compared to wild-type interactomes. The x axis demonstrates the $-\log_2$ fold-change of RNA interactome capture/whole cell extract ratios in *dis3-54* compared to wild-type interactomes. *S. pombe* Ran cycle proteins identified were plotted with circles showing Sbp1 (purple) and Spi1 (red). **B)** Alignment of Ran-binding protein (RanBP) orthologues. Protein sequence of *S. pombe* Sbp1 was aligned to its known orthologue Yrb1 in *S. cerevisiae*, and RanBP1 in *H. sapiens*. Conserved residues in all three orthologues are annotated with blue highlight and predicted α -helix or β -sheet structures annotated as green highlight or pink highlight, respectively. Numbers corresponding to amino acids (aa) are indicated. Created using Promals3D.

Exogenous Sbp1 has been shown to localise predominantly to the cytosol, consistent with the predicted cellular localisation from RanBPs (Novoa et al. 1999). Interestingly, the Ran-binding domain alone was shown to localise to the nucleus, a phenotype that has also been observed in RanBP1 (Novoa et al. 1999). This implies that regions excluded from the Ran-binding domain in Sbp1 mediate cytoplasmic localisation, notably the amino-terminal residues. So far, this is the only indication that structural features may be important in mediating localisation of Sbp1 and so this still remains speculation.

Perturbations of the Ran GTPase cycle have been described previously, and characteristic phenotypes reported have included condensed chromosomes, fragmentation of the nuclear envelope, and increased number of cells with a septum (He et al. 1998, Novoa et al. 1999). Despite these identifications, the impact of disturbance of the Ran cycle on RNA export has not been assessed, and the assessment of this could be relevant considering the role of the Ran cycle in nucleocytoplasmic transport (discussed in Section 1.5.2).

Interestingly, there are some indications from the published literature that Spi1 might interact with Dis3 based on a yeast two-hybrid screen. Binding assays demonstrated the ability of Dis3 to bind both GDP-associated and GTP-associated Ran in *S. cerevisiae* and to stimulate RanGAP activity (Shiomi et al. 1997, Noguchi et al. 1996). Although enzymatic assays were not carried out to determine if this was actually Dis3 (with exonucleolytic activity), this suggests that Sbp1/Spi1 might be connected to the exosome via Dis3. Although a direct link between Sbp1 and Dis3 has not been demonstrated, Spi1 was identified in affinity capture of Dis3 (Noguchi et al. 1996). Together with the requirement of the Ran cycle in nuclear export of some ncRNA subtypes as previously discussed, the identification of a reduction in both Sbp1 and Spi1 on poly(A)⁺ RNA in *dis3-54* (Figure 1.4) suggests that the Sbp1-Spi1 complex could be required for correct ncRNA export and therefore could explain the nuclear RNA retention observed in exosome mutants.

1.9 Objectives of this work

The RNA-interactome screen produced multiple candidate proteins that may be important in the regulation of the exosome. Through following up on data generated from this screen, I aim to develop a greater understanding of the regulation of the fission yeast nuclear RNA exosome.

Firstly, the main focus of my project explores the functional connection between RNA export and RNA decay. I aim to gain a better insight into the mechanistic link between the exosome and export by investigating the function of the predicted export-related protein Sbp1 in fission yeast.

Secondly, I explore the behaviour of the exosome complex in different exosome mutants to improve the current understanding of the impact of catalytic mutants of the exosome on the regulation of the fission yeast nuclear RNA exosome.

Chapter 2

Investigating the role of Sbp1 in exosome function

2.1 Introduction

As discussed in Section 1.6, a connection between RNA export and RNA degradation has been suggested in mammals and budding yeast (Wang et al. 2019, Silla et al. 2018, Tudek et al. 2018). However, the underlying molecular mechanisms behind this link still need to be fully delineated, as well as whether a similar mechanism can be observed in fission yeast.

The comparative RNA-interactome poly(A)+ pull down method discussed in Chapter 1 identified Sbp1 as an export-related protein that was decreased on RNA in the *dis3-54* mutant. Since retention of poly(A)+ RNA is a common phenotype observed between both exosome- and export-related proteins (described in Section 1.3.1), it is reasonable to hypothesise that altered RNA binding of the export-related protein Sbp1 could be in part responsible for the retention phenotypes observed in exosome mutants. This chapter characterises this protein to investigate a potential mechanistic link between an export-related protein and the exosome in fission yeast.

2.2 Nuclear exosome mutants show export defects

Retention of poly(A)+ RNA in the nucleus has been observed in budding yeast exosome mutants (Carneiro et al. 2007, Paul & Montpetit 2016, Rougemaille et al. 2007). Therefore, to investigate whether specific mutants of the fission yeast exosome also show this phenotype, poly(A)+ FISH was performed for *rrp6* Δ and *dis3-54* mutants (Dirks et al. 2003). poly(A)+ RNA was visualised using an oligo(dT)₂₀ probe conjugated to the Alexa488-fluorophore and a DAPI stain was used to stain the nucleus and allow detection of the poly(A) signal on the nucleus.

The *rrp6* Δ mutant showed nuclear retention of poly(A)+ RNA at 25°C when compared to the dispersed signal observed in both the nucleus and cytoplasm in WT (Figure 2.1, compare image b and image a). This poly(A)+ RNA was shown to be nuclear localised, demonstrated by the poly(A)+ signal on the DAPI (Figure 2.1 image b). The *dis3-54* mutant also showed this nuclear retention at 25°C when compared to WT (Figure 2.1, compare images c and a). However, the retention observed in *dis3-54* was lower than that observed in *rrp6* Δ (Figure 2.1, compare lanes c and b). All signals observed were shown to be dependent on RNA, as RNase treatment showed no signal observed (Figure 2.1, lane d). The observed difference in strength of retention in *dis3-54* and *rrp6* Δ could be due to different preferences of Rrp6 and Dis3 for poly(A)+ RNA, consistent with previous demonstrations of differences in specificities for substrates in Dis3 and Rrp6 (Kiss & Andrulis 2010). These data suggest that exosome mutants exhibit similar RNA nuclear retention to that seen in budding yeast, supporting a conserved mechanism between decay and RNA export pathways.

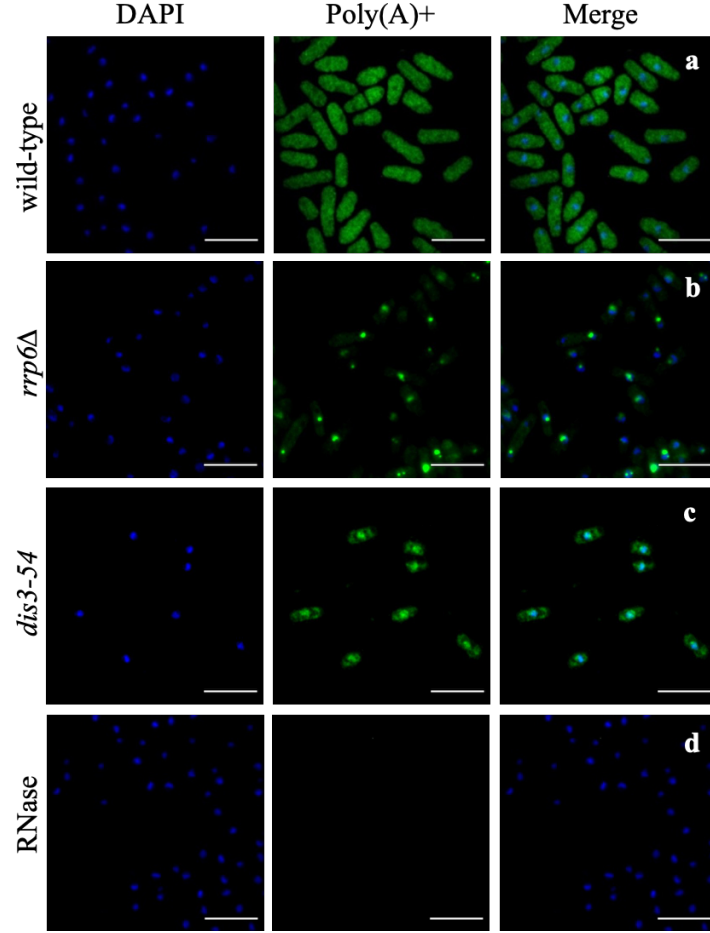


Figure 2.1: Nuclear RNA exosome mutants display poly(A)+ nuclear RNA retention. Z-stack images of poly(A)+ fluorescent in-situ hybridisation of wild-type (image a), *rrp6Δ* (image b) and *dis3-54* (image c) at 25°C. RNase treatment (+RNase, image d) demonstrates a control to show the signal is dependent on RNA. Poly(A)+ RNA was visualised with Alexa488-conjugated 5' oligo(dT)₂₀ probe (green) and DNA stained with DAPI (blue). Merged images demonstrate overlay of poly(A)+ signal on DAPI. Magnification = 100x. Scale bar = 10μm, represented by white line.

2.3 Characterisation of Sbp1

2.3.1 Biochemical purification of the native endogenous Sbp1 protein complex

To further understand Sbp1 function, native affinity purification of Sbp1 was carried out. To do this, a Flag-tagged Sbp1 strain was constructed by introducing 3xFlag to the C-terminus of Sbp1 in its genomic location by transformation using a WT *S. pombe* strain (Table 5.5). Following screening by PCR, successful insertion of the tag was confirmed by western blot using anti-Flag antibodies. Next, Sbp1-Flag was subjected to one step purification using a Flag affinity column (described in 5.4.2). Proteins from the elution fraction from the Flag affinity column were separated by SDS-PAGE electrophoresis and proteins were visualised by silver staining. Analysis of this gel revealed a large quantity of protein migrating around 25kDa (Figure 2.2) and due to its size, this was thought to be Sbp1. Further analysis of the gel identified other distinct bands and therefore the total sample was analysed by mass spectrometry (MS) to reveal the identity of these proteins that co-purified with Sbp1.

Interestingly, components of the RanGTP cycle discussed in Section 1.5.2 were identified by MS (Figure 2.2 lane 1, Table 2.1), in agreement with the predicted role of Sbp1 as a RanBP in the fission yeast Ran cycle (He et al. 1998). MS identified Sbp1 to purify with the fission yeast RanGAP Rna1 and the RanGEF Pim1 (Table 2.1). In addition, Spi1 was identified in the Sbp1 purification, in agreement with published studies (Fleig et al. 2000) (Figure 2.2 lane 1, Table 2.1). Along with the expected interactors of other fission yeast Ran cycle proteins, the MS data also identified Kap95 (Karyopherin family, importin subunit) (Table 2.1), further supporting the predicted role of Sbp1 as a shuttling protein and suggesting that Sbp1 is likely to interact with the karyopherin family of nuclear transport receptors. Interestingly, Spi1 also shows a reduced binding to poly(A)+ RNA in *dis3-54* (Figure 1.6A), suggesting that these two proteins are affected in a similar manner in the exosome mutants. Together, these data demonstrate that Sbp1 is likely to physically

interact with the shuttling protein Spi1 and with other proteins implicated in RNA transport and the RanGTP cycle (e.g. RanGAP and RanGEF), possibly forming a complex *in vivo*.

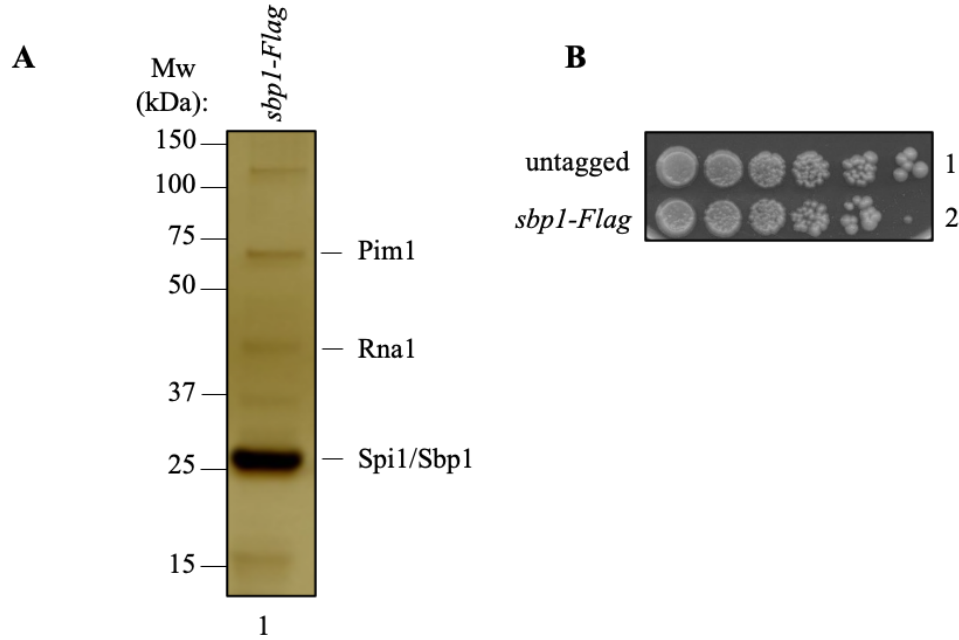


Figure 2.2: Identifying Sbp1 interacting proteins. **A)** Silver stain SDS-PAGE analysis of affinity purification of Sbp1 purified from *S. pombe*. The lane (1) represents SDS-PAGE of elution fraction from strain *sbp1-Flag*. Sample was purified using an anti-Flag M2 resin and washes and elutions were carried out with 200mM of NaCl. Mass spectrometry was carried out on total elutions from Sbp1-Flag. Lines indicate interacting proteins from mass-spectrometry analysis. **B)** Spot test growth of an untagged control (lane 1) compared to the growth of *sbp1-Flag* (lane 2). Serial cell dilutions of both strains were spotted on YES and plates were incubated for 6 days at 25°C.

Table 2.1: Mass-spectrometry results of Sbp1 affinity purification. The values indicate the intensity score of protein identified in *sbp1-Flag*.

Protein name	Intensity score
Pim1	8.28E+09
Spi1	1.13E+11
Sbp1	6.99E+10
Kap95	5.11E+10
Rna1	1.26E+10

2.3.2 Investigating Sbp1 localisation in the cell

Purification of Sbp1 revealed that Sbp1 interacts with the Ran shuttling protein Spi1, along with nuclear and cytoplasmic proteins Pim1 and Rna1, other proteins in the fission yeast RanGTP cycle, respectively. Therefore, Sbp1 cellular localisation was assessed, since RanGTP proteins can be nuclear or cytoplasmic localised. To answer this, endogenous Sbp1 was tagged by adding a green fluorescent protein (GFP) to the C-terminus of Sbp1 in its genomic location by transformation using a WT *S. pombe* strain (Table 5.5). Colonies were screened by PCR and successful insertion of the tag was confirmed by the identification of GFP expression in the cell. Live cell microscopy was then used to assess the distribution of Sbp1-GFP in WT cells. In addition to the signal observed in the cytosol, as previously described for Sbp1 (Novoa et al. 1999), the majority of Sbp1-GFP was enriched inside the nucleus (Figure 2.3A, image d). Quantification of this fluorescent signal confirmed this observation of an enriched signal in the nucleus compared to the more even signal observed in the cytoplasm (Figure 2.3C). The presence of some sort of vacuolar structures were identified in the cytoplasm and Sbp1 was excluded from these areas (Figure 2.3A, image d). This suggests either that Sbp1 is excluded from vacuoles due to a lack of a requirement in transport across vacuolar membranes (in contrast to the requirement at the nuclear envelope) or that Sbp1 is excluded due the increase in size of Sbp1-GFP compared to Sbp1 (Figure 2.3).

The GFP tag added was relatively large (around 25kDa) and so since Sbp1 is a Ran component, it was predicted that the result seen could be due to the large size of the GFP tag interfering with the function of Sbp1 and thus leading to alterations in Sbp1 localisation. To exclude this possibility, a spot test was carried out in which serial dilutions were plated on YES media and plates were incubated at 25°C to compare the growths of the untagged strain and *sbp1-GFP*. Indeed, the spot test showed that the growth of *sbp1-GFP* was comparable to the untagged strain (Figure 2.3B, compare lane 2 to 1), implying that the tag did not interfere with the function.

These data suggest that Sbp1 is likely to shuttle from the nucleus to the cytoplasm as previously reported for other Ran protein orthologues (Kunzler et al.

2000). Altogether, these results suggest that Sbp1 plays a role in the Ran cycle due to its ability to act as a shuttling protein.

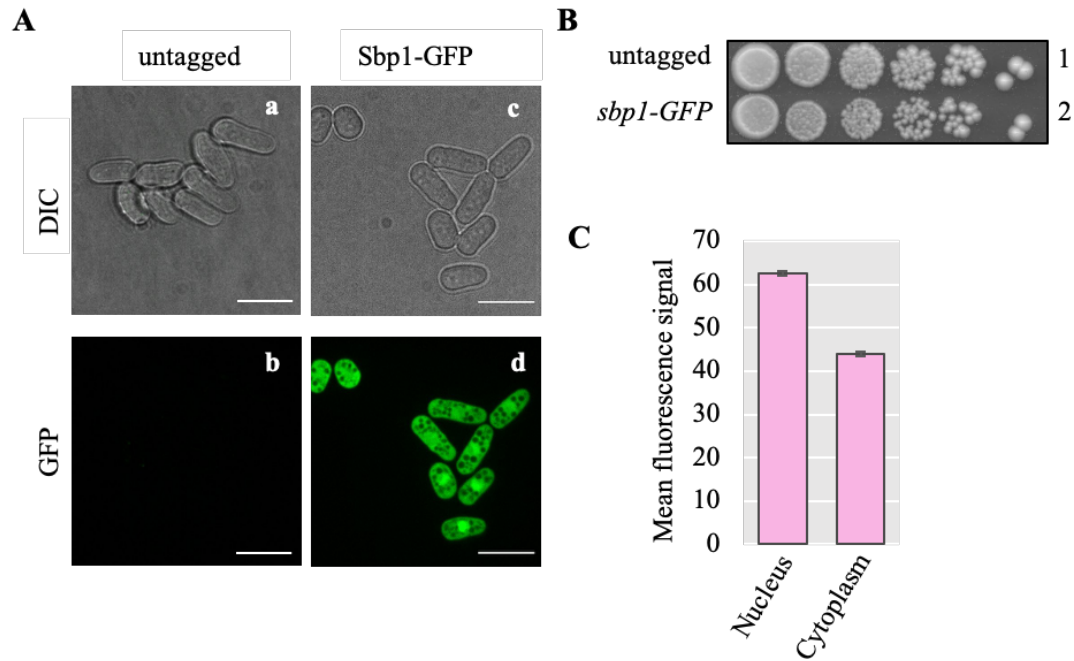


Figure 2.3: Sbp1 is localised in the nucleus and the cytoplasm. **A)** Live-microscopy shows an untagged control (images a-b) and fluorescent labelled Sbp1 (GFP) in a wild-type background (images c-d). Magnification = 100x. Scale bar = 10 μ m, represented by a white line. Images shown are from the differential interference contrast (DIC) filter showing the outline of the cell (images a, c) and from the green fluorescent protein (GFP) filter (images b, d). All images were taken at 25°C. **B)** Spot test growth of an untagged control (lane 1) compared to the growth of *sbp1-GFP* (lane 2). Serial cell dilutions of both strains were spotted on YES and plates were incubated for 6 days at 25°C. **C)** Quantification of mean fluorescent GFP signal in *sbp1-GFP*. ImageJ was used to measure the mean fluorescent signal provided by the GFP tag in the nucleus and cytoplasm. Error bars = $\pm$ SE.

2.4 Functional analysis of Sbp1

2.4.1 Generation of a temperature sensitive (ts) mutant of Sbp1

To further investigate Sbp1 function in RNA regulation, a genetic approach was employed to create a temperature sensitive (ts) allele of Sbp1. Impairing Sbp1 function by deletion of Sbp1 was not possible since Sbp1 deletion has been reported to be inviable (Kim et al. 2010, Novoa et al. 1999). To impair Sbp1 function, the Sbp1 open reading frame (ORF) and the 3' untranslated region (UTR) were cloned into an acceptor pFa6a Kanamycin plasmid (Table 5.3) and error prone mutagenesis was carried out on the ORF + 3'UTR regions using unfavourable PCR conditions combined with a low fidelity polymerase. The resulting mutagenised product was transformed into a *S. pombe* WT background (Table 5.5) and screened for kanamycin resistance and for sensitivity to low and high temperatures. The ts colonies identified were isolated from the ts strains observed on phloxine plates. Phloxine was used to identify which strains were sick compared to WT, and therefore to identify which strains might have an impairment in Sbp1 function. This could be identified by a visual difference in colour between healthy and sick cells growing on the phloxine plates, as WT cells are pale pink on phloxine plates due to being able to eliminate phloxine, whilst sick cells retain the phloxine and are therefore dark pink in colour (Sabatinos & Forsburg 2010). Using this approach, several colonies were chosen for further analyses to identify the mutations responsible for the ts phenotype. Mutations were identified by PCR amplification and sequencing using Sbp1 specific primers (Table 5.1).

Candidate strains were sequenced to identify mutations and the ts phenotype was further confirmed by assessing their growth at different temperatures. Out of the candidate colonies, one strain was identified by spot test to not be able to grow at 37° (Figure 2.4A), identifying that this strain is ts. Considering that Sbp1 is an essential protein, this indicated that Sbp1 function was impaired in this strain and therefore this strain was chosen for further analyses and was called *sbp1-1*. Sequencing

revealed a T to C nucleotide substitution at position 688 (T668C), resulting in a single point mutation: a tryptophan to arginine substitution at position 166 (W166R) (Figure 2.4B, red star in RBD). Based on the known structure of the orthologues (Figure 1.6B), the budding yeast orthologue Yrb1 and the mammalian orthologue RanBP1, this domain is predicted to be the Ran-binding domain (Figure 2.4C), a domain shown to be important for cellular Sbp1 localisation (Novoa et al. 1999).

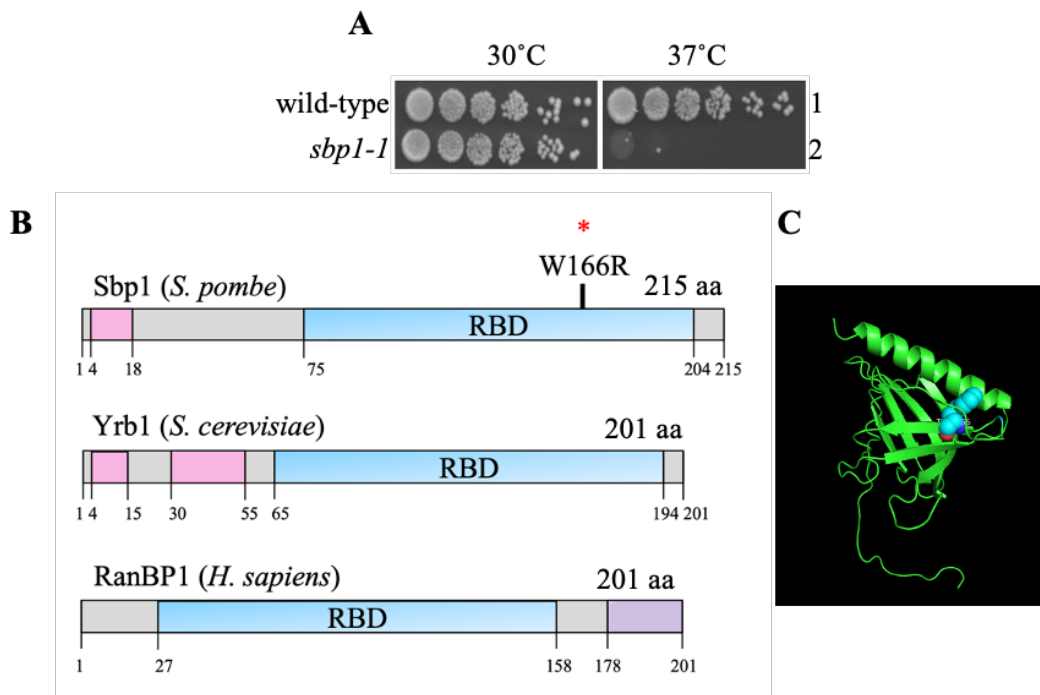


Figure 2.4: Generation of *sbp1-1* temperature sensitive mutant. **A)** Spot test showing serial dilutions of wild-type (lane 1) and *sbp1-1* (lane 2). Cells were spotted on YES and incubated at 30°C and 37°C. Loss of growth in *sbp1-1* at 37°C highlights temperature sensitivity. **B)** Schematic structure of Ran-GTPase binding proteins in different organisms: Sbp1 (*S. pombe*), Yrb1 (*S. cerevisiae*) and RanBP1 (*H. sapiens*). Colours represent domains: Ran-binding domain (RBD, light blue), coiled-coil (purple), and low complexity regions (pink). Numbers indicated below the diagram correspond to amino acid (aa) positions. *sbp1-1* W166R mutation is highlighted in the predicted RBD of *S. pombe* Sbp1 structure (*). Domain representations created using SMART based on sequences from UniProt IDs: *S. pombe* Q09717, *S. cerevisiae* P41920, *H. sapiens* P43487. **C)** Location of W166R mutation (coloured spheres) on the predicted structure of Sbp1 (green) based on the Ran-binding protein orthologues. Created with Phyre2 (Kelley et al. 2015).

2.4.2 Cellular localisation of poly(A)+ RNA is affected in a *sbp1-1* mutant

Next, since Sbp1 was shown to interact with members of the Ran cycle (Figure 2.2, Table 2.1), the Sbp1 ts mutant (*sbp1-1*) was used to assess whether Sbp1 is indeed involved in RNA transport. To test this hypothesis, global poly(A)+ RNA FISH was carried out using the same probes as in Section 2.2 (Alexa488 fluorophore to detect polyadenylated transcripts and DAPI staining to locate the nucleus). Cells were grown at 25°C, followed by a 4 hour shift of WT and *sbp1-1* at the restrictive temperature (37°C) of ts *sbp1-1* (Figure 2.4A). A 4 hour shift was chosen as this time proved sufficient in inducing a phenotype of an alteration in poly(A)+ FISH in microscopy when compared to WT, whilst minimising the quantity of dead cells visible by microscopy. Poly(A)+ RNA FISH showed a homogenous distribution of poly(A)+ RNA comparable to WT in *sbp1-1* at 25°C (Figure 2.5). However, greater nuclear signal appears in *sbp1-1* after 4 hours at 37°C (Figure 2.5A, white arrows).

Quantification of the signals in the nucleus and cytoplasm was carried out by calculating the median fluorescence in both cellular compartments (nucleus and cytoplasm) and calculating the ratio of the median fluorescence in the nucleus to that in the cytoplasm. This revealed a slight increase of signal coming from the nucleus, suggesting an accumulation of polyadenylated RNA in *sbp1-1* (Figure 2.5B). This is in accordance with the observation of poly(A)+ nuclear retention in *S. cerevisiae* RanGEF and RanGAP mutants (Forrester et al. 1992). These data suggest that RNA export might be compromised in *sbp1-1* when cells grow at restrictive temperature, in agreement with the possible role of Sbp1 in RNA transport. Further analysis is needed to untangle whether Sbp1 contributes to regulation of mRNAs or ncRNAs. To determine which type of poly(A)+ RNA could be contributing to this effect, localisation of individual transcripts should be assessed using a single molecule fluorescent approach, such as sm FISH.

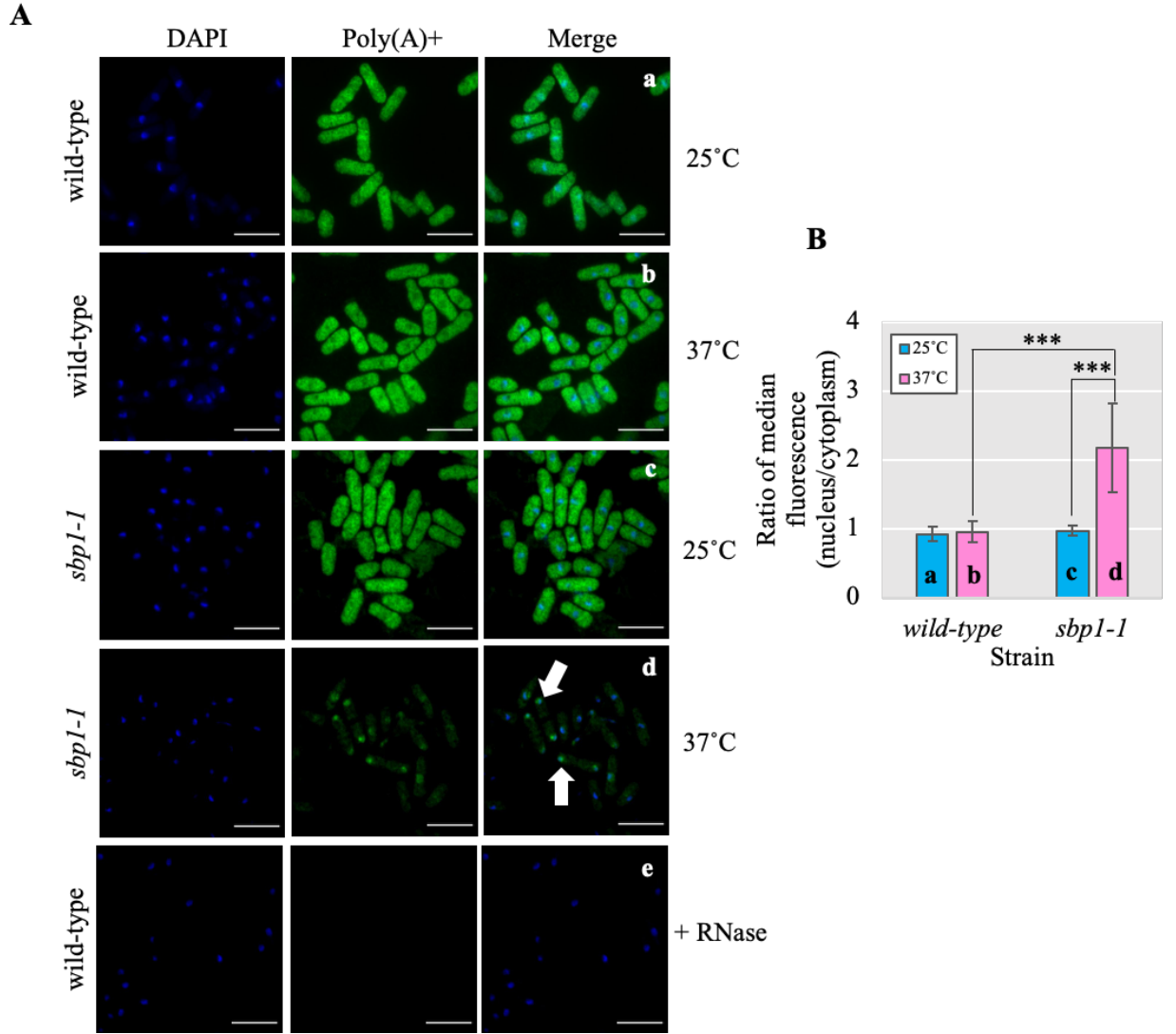


Figure 2.5: *sbp1-1* has an export defect at restrictive temperature. **A)** Z-stack images of poly(A)+ Fluorescent In-Situ Hybridisation of wild-type (images a-b) and *sbp1-1* (images c-d) after 4 hours at permissive (25°C) or restrictive (37°C) temperatures. Merged images of poly(A)+ (green) and DAPI nuclei staining (blue). Magnification = 100x. Scale bar = 10µm and is represented by a white line. White arrows point to poly(A)+ retention localised on DAPI signal. Treatment with RNase channel represents a negative control (image e). **B)** Quantification of fluorescence ratio of wild-type (bars a-b) and *sbp1-1* (bars c-d) at 25°C (blue) and 37°C (pink). A fixed area was taken on the DAPI signal to correspond to the nucleus and the DIC images (not shown) were used to take the same fixed area in the cytoplasm. ImageJ was used to measure the median signal in this fixed area which is displayed as a ratio between the nucleus and cytoplasm (nucleus/cytoplasm). These results were used for statistical analysis, demonstrating a significant effect of strain ($< 2e-16$ ***) and temperature (0.000658 ***) on median fluorescence (pair-wise ANOVA comparison test). Significance codes: *** = < 0.001 . Error bars = standard deviation.

2.4.3 Genetic interactions between the exosome and Sbp1

Both *dis3-54* and *sbp1-1* mutants show retention of poly(A)⁺ RNA in the nucleus (Figure 2.5). Therefore, a genetic interaction between *sbp1-1* and the exosome was assessed to test the relationship between Sbp1 and the exosome. To do this, a spot test was carried out by plating serial dilutions of WT, *sbp1-1*, and exosome mutants (*rrp6Δ* and *dis3-54*) on YES media and incubating the plates at 30°C, 34°C and 37°C. 30°C was used as a control, as previous experiments showed *sbp1-1* to grow to levels comparable to WT (Figure 2.4A). It was thought that a genetic interaction between *sbp1-1* and exosome mutants would be hard to identify at 37°C, considering that *sbp1-1* is ts. Therefore, plates were also incubated at 34°C as *sbp1-1* was identified as viable at this temperature.

A synthetic lethality was observed when the *sbp1-1* mutation was combined with exosome mutants at 34 °C, demonstrated by the reduced growth of *sbp1-1 dis3-54* or *sbp1-1 rrp6Δ* double mutants (Figure 2.6, compare rows 3 and 5 or 4 and 6 respectively). This was noticeable at 34°C despite *sbp1-1* growing similarly to WT at this temperature. These results indicated that Sbp1 genetically interacts with the exosome and highlighted a connection between Sbp1 function and the exosome.

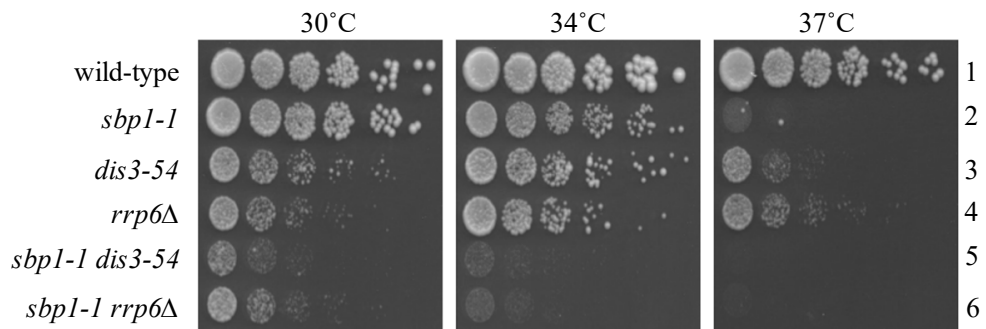


Figure 2.6: ***sbp1-1* has a negative genetic interaction with exosome mutants.** Serial cell dilutions of wild-type (lane 1), *sbp1-1* (lane 2), *dis3-54* (lane 3), *rrp6Δ* (lane 4), and double mutants of *sbp1-1* with *dis3-54* (lane 5) or *rrp6Δ* (lane 6) were spotted on YES and plates were incubated for 6 days at indicated temperatures. Numbers on right-hand side correspond to each row.

To understand whether the additive synthetic phenotype on growth observed in the double mutants could be due to de-regulated RNA localisation, poly(A)+ FISH was performed on *sbp1-1* and *sbp1-1* combined with *dis3-54* or *rrp6Δ*.

An additive effect was observed in the *sbp1-1 dis3-54* double mutant at 25°C (Figure 2.7A, image i). However, this additive effect was not as visually striking in the *rrp6Δ sbp1-1* double mutant (Figure 2.7A, image k) at the same temperature. Quantification of this signal was again carried out by calculating the median fluorescence in both cellular compartments (nucleus and cytoplasm) and calculating the ratio of the median fluorescence in the nucleus to that in the cytoplasm. This quantification confirmed the additive effect seen in the *sbp1-1 dis3-54* double mutant at 25°C (Figure 2.7B, bar i) with a significant increase in fluorescence when *sbp1-1* is compared to *sbp1-1 dis3-54* at 25°C (p adjusted = < 0.001). This additive effect suggests that Sbp1 and the exosome can act in pathways that are independent but complementary. Firstly, this set of data suggests that the exosome and Sbp1 may be connected at the molecular level. Secondly, this suggests that Sbp1 can act in a different manner with specific subunits of the nuclear exosome, shown by the different outcomes observed between the two subunits in FISH.

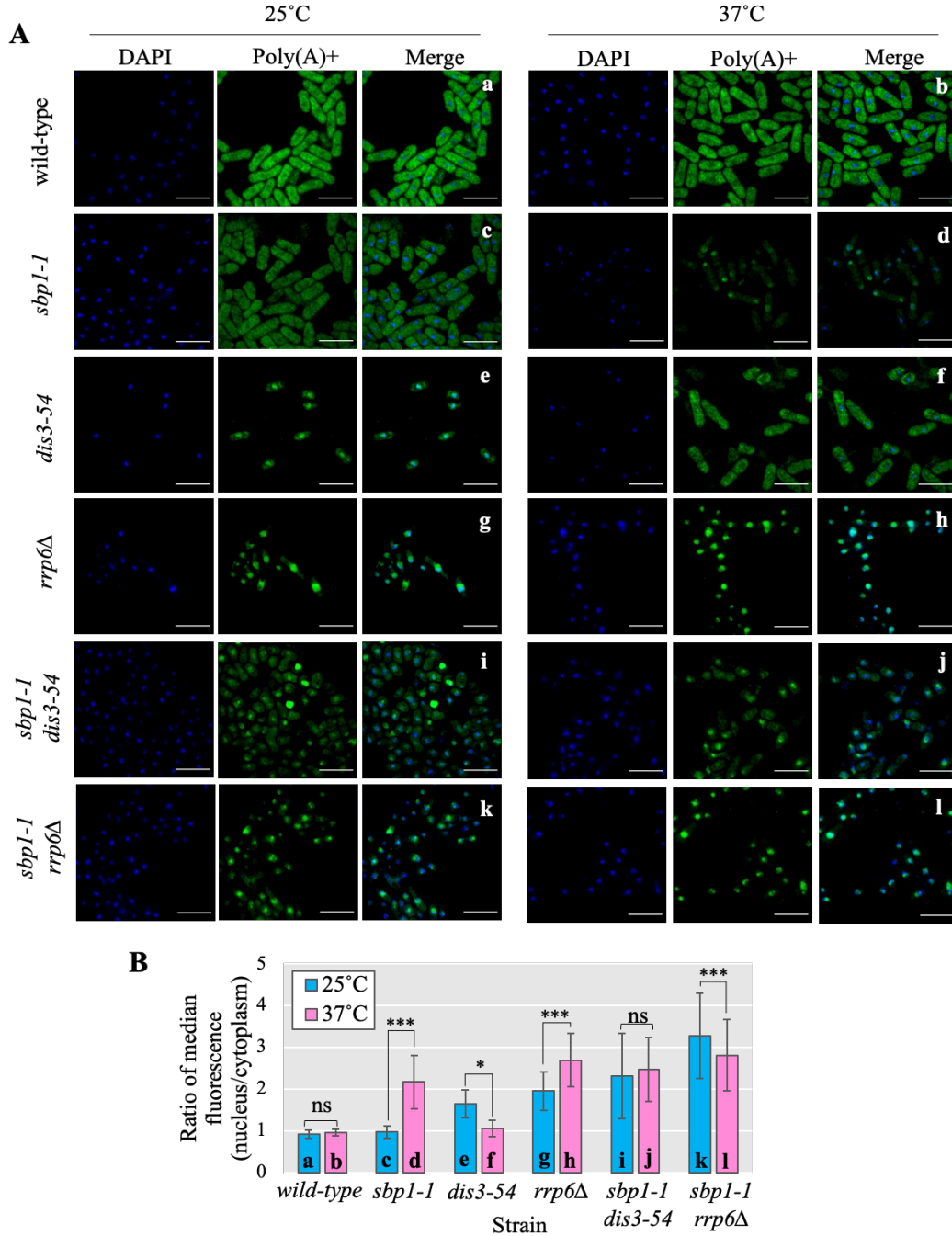


Figure 2.7: Nuclear RNA retention is enhanced in the *sbp1-1 dis3-54* double mutant.

A) Z-stack images of poly(A)+ fluorescent in-situ hybridisation of *sbp1-1* (c-d), *dis3-54* (e-f), *rrp6Δ* (g-h) and *sbp1-1* with *dis3-54* (i-j) or *rrp6Δ* (k-l) after 4 hours at (25°C) or (37°C). Images shown are staining of poly(A)+ RNA (green), nuclei (DAPI, blue) and merged. Magnification = 100x, scale bar = 10μm, represented by a white line. **B)** Quantification: A fixed area was taken on the nucleus (using DAPI images) and the cytoplasm (using DIC, not shown). ImageJ calculated the median signal in both fixed areas which was used to calculate a ratio between the nucleus/cytoplasm. A significant effect of strain ($< 2e-16$ ***) and temperature (0.000658 ***) (25°C (blue) and 37°C (pink)) was observed on fluorescence (pair-wise ANOVA). Significance codes shown represent those only for intra-strain results. Sig. codes: *** = < 0.001 , ** = $0.001 < 0.01$, * = $0.01 < 0.05$, ns = not significant. Error bars = $\pm$ standard deviation.

2.5 Transcriptome analysis of *sbp1-1*

An RNA sequencing (RNA-seq) approach was employed to gain a greater insight into how Sbp1 contributes to RNA regulation and to assess how the transcriptome is affected in Sbp1 mutants. To address this, RNA-seq was performed using ribo-depleted total RNA in WT, *sbp1-1*, and *dis3-54*. The experiment was carried out with samples grown at 25 °C overnight, followed by RNA extraction after a 4 hour shift to 37 °C, with the reasoning behind this 4 hour shift described in Section 2.4.2. To assess the differences, differential gene analysis was performed using DESeq2 package (Love et al. 2014). Integrated genome browser (IGB) was used for single gene analysis, using the Cramer annotation (Eser et al. 2016) to visualise the transcripts indicated as differentially expressed and therefore to visually assess the impact of the *sbp1-1* mutation.

The quality of the sequencing made analysis of this RNA sequencing very difficult. Firstly, the depth of the transcriptome coverage was very poor when compared to the expected coverage. Although many highly expressed genes could be detected, this vastly impacted the detection of poorly expressed genes and therefore it is predicted that this resulted in missing values. Secondly, differential gene analysis identified a high enrichment in transcripts described as encoding for ‘ribosome-associated proteins’ in the down-regulated gene set, indicating that this RNA-sequencing was subjected to ribosomal contamination and that the ribo-depletion step was not successful in the control (WT). If ribo-depletion was not successful in WT then an identified decrease in transcripts encoding for ribosome-associated proteins in *sbp1-1* and *dis3-54* would appear as false positives. Further, comparing the differential analysis between the transcripts identified as differentially expressed in this data set and the poly(A)+ RNA pull down method in *dis3-54* (Birot et al. unpublished manuscript from the Vasilieva lab, 2020, Kilchert et al. BioRxiv, 2019, accepted to Genome Research 2020) proved difficult. Firstly, only 124 transcripts were identified as up-regulated (fold change > 1.5 and p-value < 0.05) in *dis3-54* in this study (discussed later, Figure 2.8A), compared to 201 transcripts satisfying the same criteria in the poly(A)+ pull down method (Birot

et al. unpublished manuscript from the Vasilieva lab, 2020, Kilchert et al. BioRxiv, 2019, accepted to Genome Research 2020). Further, the RNA-seq carried out in the poly(A)+ pull down method (Biro et al. unpublished manuscript from the Vasilieva lab, 2020, Kilchert et al. BioRxiv, 2019, accepted to Genome Research 2020) was poly(A)+ RNA-seq, but the RNA-seq carried out in this study was total RNA. As well as further complicating comparison between these data sets, this also demonstrates the low coverage observed in this study as the identification of a greater number of transcripts would be expected in total RNA-seq compared to RNA-seq of poly(A)+ RNA only. Out of the 124 transcripts identified as up-regulated in *dis3-54* (discussed later, Figure 2.8A), only 57 of these were identified in the poly(A)+ pull down method. Comparison of these data sets was further complicated by the high proportion of unannotated ncRNAs (35 out of 124) in this data set identified from the annotation of Eser et al. (2016). Taken together, this highlights many of the issues encountered in this approach and suggests that these issues need to be considered when analysing the data. It is therefore necessary to confirm expression profiles by northern blot before conclusions can be drawn.

2.5.1 Up-regulation of ncRNA transcripts observed in *ts sbp1-1*

Analysis identified RNAs that were shown to be up-regulated in *sbp1-1* and *dis3-54* at 37°C but that were not up-regulated in WT (to exclude the possibility that these genes are up-regulated due to heat shock). Analysis revealed that 87 transcripts were up-regulated in *sbp1-1* and 124 up-regulated in *dis3-54* (fold change > 1.5 with a p-value < 0.05) (Figure 2.8A). Out of these transcripts, 44 and 46 were up-regulated ncRNAs in *sbp1-1* and *dis3-54* respectively, demonstrating a high proportion of ncRNAs out of total RNAs for both datasets (Figure 2.8B). Interestingly, most of the total transcripts up-regulated in *sbp1-1* were also up-regulated in *dis3-54* and this was also observed for up-regulation of ncRNAs (Figure 2.8, compare overlapping circles of A to B). Examples of specific ncRNAs up-regulated for both *sbp1-1* and *dis3-54* include *SPNCRNA.1114* (encoding for *ncRNA.1114*), *SPNCRNA.103*

(encoding for *sme2*), and *SPSNORNA.43* (encoding for *snR91* snoRNA) (Figure 2.9A, compare pink and blue to green). *sme2* is a ncRNA involved in sequestering Mmi1 during sexual differentiation (Ding et al. 2012), further suggesting a link between Sbp1 and the exosome. Similarly, both *ncRNA.1114* and *sme2* were shown to be up-regulated in RNA-seq from the *dis3-54* poly(A)+ pull down method described previously (Biot et al. unpublished manuscript from the Vasilieva lab, 2020, Kilchert et al. BioRxiv, 2019, accepted to Genome Research 2020).

Northern blot was performed to identify the levels of the ncRNAs *ncRNA.1114* and *sme2* and to confirm these expression profiles. The data from the northern blots confirmed the increase in levels of both ncRNAs in *sbp1-1* and *dis3-54*. Firstly, levels of ncRNA *ncRNA.1114* were shown to be increased in *sbp1-1* and *dis3-54* compared to WT (Figure 2.9B, compare lane 2 and lane 3 to lane 1). Secondly, levels of the ncRNA *sme2* were shown to be increased in both *sbp1-1* and *dis3-54* compared to WT (Figure 2.9B, compare lane 2 and lane 3 to lane 1). Both northern blots confirm the expression profiles from RNA-seq (Figure 2.9, compare B and C to the corresponding profile in A).

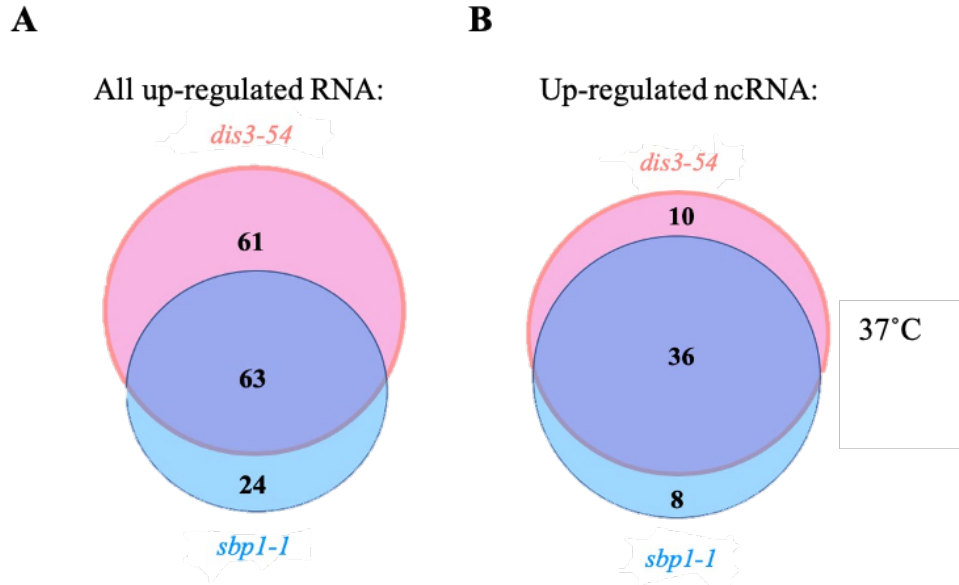


Figure 2.8: **Venn diagrams of common transcripts up-regulated in *sbp1-1* and *dis3-54*.** Venn diagrams made from RNA sequencing data of all up-regulated transcripts (**A**) and up-regulated ncRNA transcripts (**B**) that satisfied the condition of p adjusted value < 0.05 and log fold change >1.5. For both sets *sbp1-1* is shown in blue and *dis3-54* is shown in pink. Area of overlap demonstrates shared transcripts between *sbp1-1* and *dis3-54*. All data are from results at 37°C, compared to wild-type at 37°C. Venn diagrams made with Biovenn (Hulsen et al. 2008).

These data show that Sbp1 is important in controlling the levels of selected non-coding transcripts, which are also regulated by Dis3, suggesting that Sbp1 and Dis3 might be part of the same mechanism. It is not clear whether up-regulated levels of nc transcripts are connected to nuclear retention observed in Sbp1 mutants (Figure 2.5) since the cellular localisation of these transcripts can not be determined from the RNA-seq data. Further, the northern blot confirmed that up-regulated ncRNAs are characterised as unstable ncRNAs, a subtype that is usually nuclear as previously mentioned. If the phenotypes of retention (Figure 2.5) and up-regulation (Figure 2.9B) are indeed caused by the same subtype of RNA, this would suggest that these unstable ncRNAs are usually exported in a manner that is dependent on Sbp1 and therefore that this function is impaired in *sbp1-1*. However, components of the Ran cycle and Crm1 are linked to the export of stable ncRNAs such as nuclear RNAs (sn/snoRNAs) (discussed in Section 1.5.2), suggesting that the retention observed previously (Figure 2.5) is more likely to be due to defects in the export of

other stable ncRNAs. The identification of up-regulation of *snR91* demonstrates a subtype that is more likely to contribute to the retention observed in FISH, although this needs to be confirmed by northern blot. I conclude that further analysis is required to untangle whether the ncRNAs identified in *sbp1-1* are nuclear or cytoplasmic localised and to identify whether the two phenotypes (retention in FISH and up-regulation of ncRNA in RNA-seq) are linked or independent.

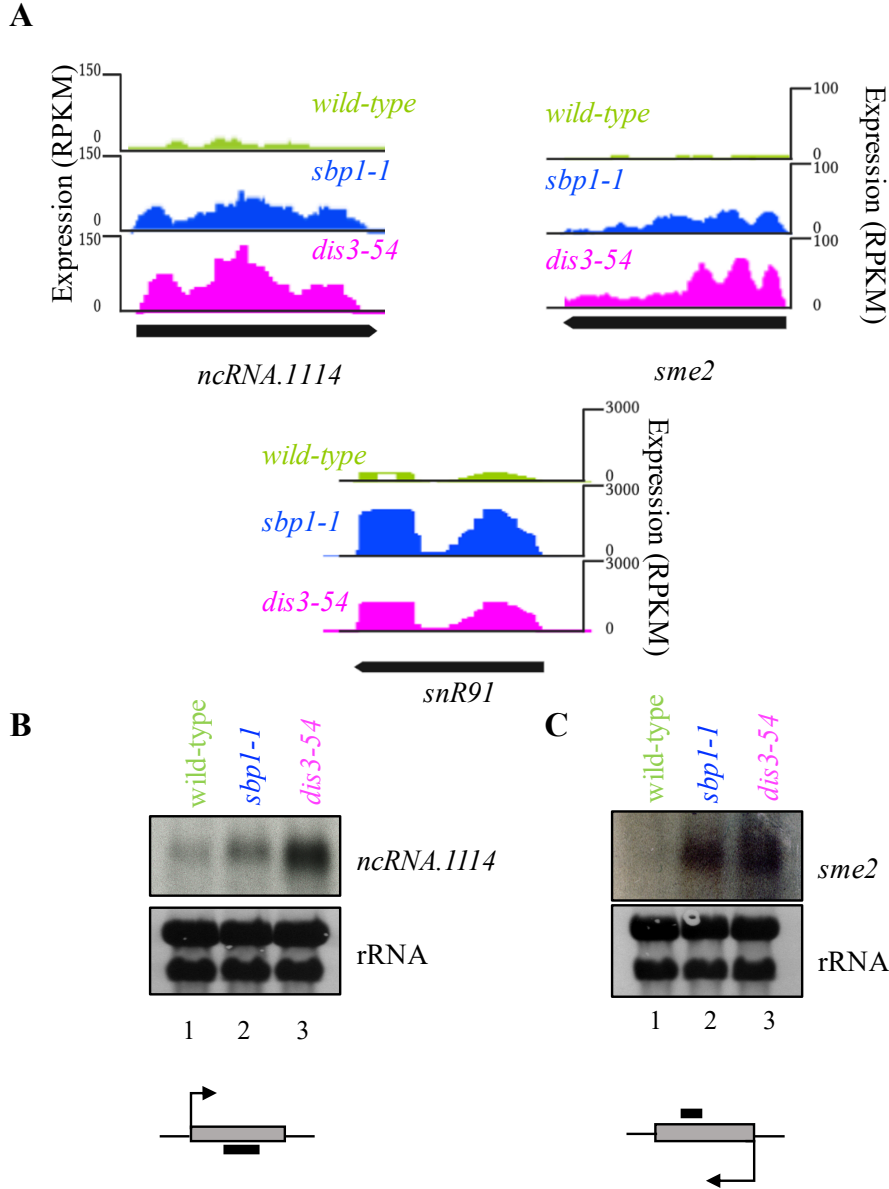


Figure 2.9: **ncRNA up-regulation in *sbp1-1* and *dis3-54*.** **A)** Strand-specific total ribo-depleted RNA-sequencing of *sbp1-1* (blue) and *dis3-54* (pink) compared to wild-type (green). Expression of transcripts (*ncRNA.1114*, *sme2*, and *snR91*) is shown in reads per kilobase of transcript per million mapped reads (RPKM) and normalised to library size. The black schematic underneath each graph illustrates the composition of the gene and strand orientation. Confirmation of expression profiles by northern blot for *ncRNA.1114* (**B**) and *sme2* (**C**). For both, RNA was extracted from wild-type (lanes 1, green), *sbp1-1* (lanes 2, blue) and *dis3-54* (lanes 3, pink). Methylene blue-stained rRNA bands represent loading control. Schematics underneath each blot illustrates the area spanning the probe. All results were from RNA extracts from cells grown at 25°C and shifted to 37°C for 4 hours and were carried out in duplicate.

2.6 The nuclear exosome localisation is preserved in *sbp1-1*

Next, the localisation of the exosome was assessed to identify whether Sbp1 might affect levels of selected exosome substrates by contributing towards exosome localisation. To answer this question, the helicase Mtl1 and exonuclease Rrp6 were tagged with mCherry in WT and an *sbp1-1* background, to be able to visualise their localisation *in vivo*. Strains were screened for temperature sensitivity along with genomic sequencing of the region spanning the T668C mutation (to confirm *sbp1-1*) and mCherry fluorescence was used to confirm the presence of fluorescently tagged exosome. The resulting strains were used for fixed-microscopy, after a 4 hour shift as previously described to induce *sbp1-1*, with the aim of capturing any alterations in exosome localisation *in vivo* when Sbp1 is impaired.

Both Rrp6 and Mtl1 were shown to form discrete nuclear foci in a WT background as expected (Figure 2.10, refer to images a and e). The signal for Mtl1 is more dispersed than that identified for Rrp6 (Figure 2.10, refer to images e and a). The localisation of Rrp6 or Mtl1 did not seem to be altered in a *sbp1-1* background after 4 hours at the restrictive temperature of *sbp1-1* (Figure 2.10, compare images d to b and h to f respectively). Since Sbp1 was proposed to interact with Dis3 via Spi1, this approach should be repeated on fluorescently labelled Dis3 to assess whether this observation is consistent for Dis3. From these data, it can be concluded that Sbp1 does not alter the localisation of the exosome.

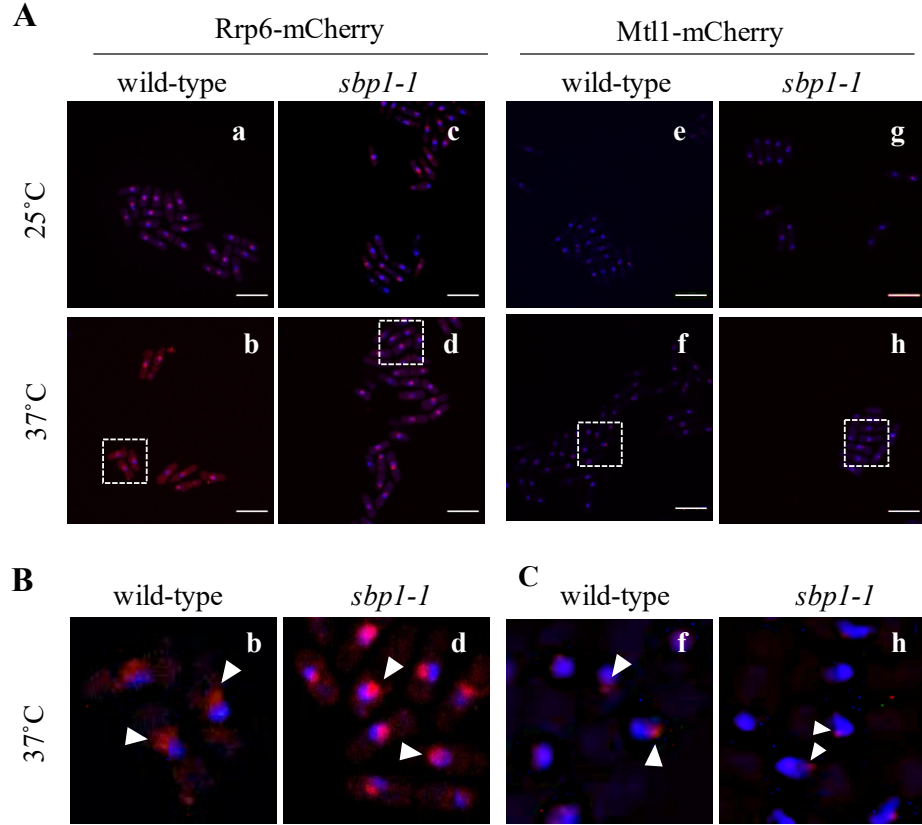


Figure 2.10: Localisation of exosome subunits is not altered in *sbp1-1*. **A)** Fixed-microscopy shows mCherry fluorescently-labelled Rrp6 (images a-d) or Mtl1 subunits (images e-h) of the exosome in both wild-type and *sbp1-1* backgrounds. Images shown are merged mCherry (red) and DAPI (blue) channels after 4 hours at *sbp1-1* permissive (25°C) or restrictive (37°C) temperatures. Magnification = 63x, scale bar = 10 μ m, represented by a white line. Dashed boxes show the area corresponding to the enlarged image. **B)** Enlarged image of Rrp6-mCherry in *sbp1-1* at 37°C. **C)** Enlarged image of Mtl1-mCherry in *sbp1-1* at 37°C. White arrows highlight Rrp6 (**B**) or Mtl1 (**C**) localisation on the nuclear DAPI signal. Images shown are merged mCherry (red) and DAPI (blue) channels.

2.7 Aberrant Sbp1 localisation is seen in the exosome mutants

Next, Sbp1 localisation was assessed in exosome mutant backgrounds to investigate whether mutations in the exosome could alter the normal localisation of Ran cycle proteins and thus explain the genetic links observed between the exosome and proteins of the Ran cycle (Figure 2.6). It was hypothesised that any alteration to the normal distribution of Ran cycle proteins could partially explain the reduced occupancy of Ran proteins in exosome mutants (Sbp1 and Spi1) (Figure 1.6).

To answer this question, the *sbp1-GFP* strain constructed previously was introduced into three exosome mutant backgrounds (*dis3-54*, *mtl1-1*, and *rrp6Δ*) by random spore analysis. Strains were confirmed by screening for fluorescence (for Sbp1) and sequencing to confirm the mutations in the exosome proteins. As discussed previously, Sbp1 was found to be localised in both cellular compartments with a slightly higher signal observed in the nucleus. Interestingly, a strong increase of Sbp1-GFP signal was observed in the nucleus in *dis3-54* (Figure 2.11, image b), *mtl1-1* (Figure 2.11, image c), and *rrp6Δ* (Figure 2.11, image d) when all compared to wild-type (Figure 2.11, image a).

Quantification of fluorescent signal of the GFP tag showed a 1.2x, 1.4x, and 1.2x increase in Sbp1-GFP signal in the nucleus compared to the cytoplasm (p adjusted < 0.001) for *dis3-54*, *mtl1-1*, and *rrp6Δ* respectively (Figure 2.11). As previously, spot test growth of the GFP tagged Sbp1 in *dis3-54*, *mtl1-1*, and *rrp6Δ* backgrounds was carried out to ensure that the change in the fluorescent signal observed was not due to an impairment in growth caused by the GFP tag in these backgrounds (same as in Figure 2.3B). Spot test growth of all strains was comparable to the growth of the untagged strain (Figure 2.3B), implying that the tag did not interfere with the function.

It could be possible that alterations to the normal localisation of Sbp1 in *dis3-54*, *mtl1-1*, and *rrp6Δ* could disrupt the role of Sbp1 in the Ran cycle and therefore alter RNA export. This disruption could decrease the ability of Sbp1 to bind to RNA in

an exosome mutant background and thus could be a reason for the nuclear retention observed in exosome mutants (Figure 2.1). Taken together, these results suggest that the normal localisation of the RanBP Sbp1 is altered in exosome mutants, and so that the distribution of other Ran associated proteins may also be altered.

Lastly, Sbp1 abundance in an exosome-mutant background was assessed to make sure that the altered localisation and RNA binding of Sbp1 in *dis3-54* (Figure 1.5A) are not due to changed protein levels of Sbp1 in an exosome mutant background (*rrp6Δ*, *mtl1-1*, and *dis3-54*). To address this question, proteins were extracted from tagged Sbp1 in a WT background (*sbp1-GFP*) and Sbp1 tagged in the exosome mutant backgrounds (*sbp1-GFP dis3-54*, *sbp1-GFP rrp6Δ*, and *sbp1-GFP mtl1-1*) all grown at 25°C. Proteins were separated by SDS-PAGE and Sbp1 abundance was assessed for by the use of an anti-GFP antibody and ponceau staining was used to control for loading. Sbp1 abundance was visibly reduced in *dis3-54* when compared to WT, a phenotype that was unique to *dis3-54* (Figure 2.12A). This could indicate that the enhancement in nuclear fluorescence of Sbp1 in *dis3-54* (Figure 2.11, image b) is due to a reduced signal in the cytoplasm, which would result in an increase in contrast between these compartments and thus would seem visually enhanced in the nucleus. Nonetheless, it is clear that Sbp1 protein abundance is altered in a *dis3-54* background and this provides further support for a link between Sbp1 and Dis3.

Considering the alteration in Sbp1 abundance in the *dis3-54* background, protein abundance of Dis3 in *sbp1-1* was assessed to identify whether a similar phenotype could be seen when Sbp1 function is impaired. To do this, proteins were extracted from cells grown at 25°C and shifted to 37°C for 4 hours. Proteins were separated by SDS-PAGE electrophoresis and Dis3 abundance was assessed by western blot using an antibody against Dis3 (Table 5.4). This identified that, although Sbp1 abundance was altered in a *dis3-54* background (Figure 2.12A), Dis3 abundance was not altered in a *sbp1-1* background. Taken together, these results show that the exosome may play a role in the cellular localisation of Sbp1, suggesting that a functional exosome could be necessary for proper localisation of Ran components. Further, the reduction in protein levels of Sbp1 in a *dis3-54* background could explain the export defect observed in *dis3-54*, as less Sbp1 is available for its role in the Ran cycle.

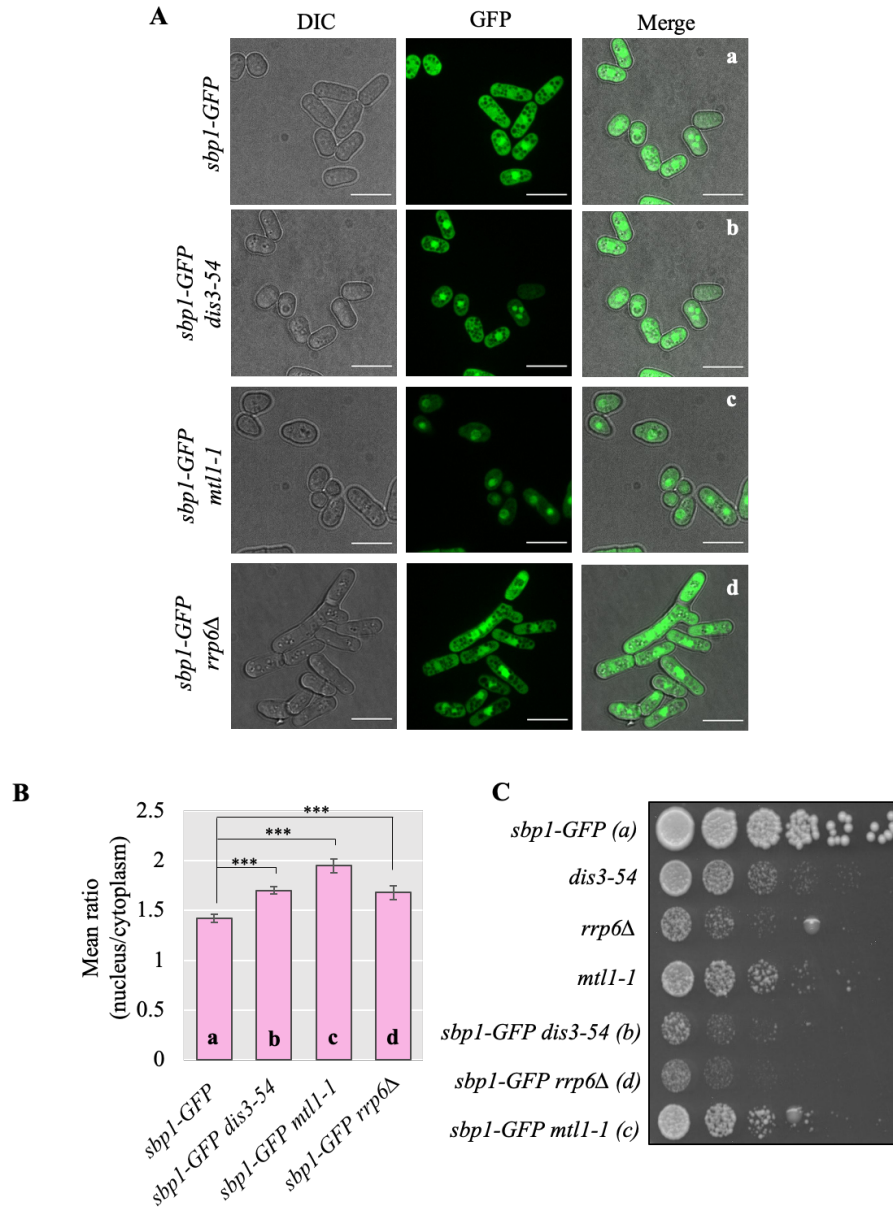


Figure 2.11: Aberrant Sbp1 localisation in exosome mutants. **A)** Sbp1 was tagged with GFP and in wild-type (image a), *dis3-54* (image b), *mtl1-1* (image c), and *rrp6Δ* (image d) backgrounds. Live-cell microscopy identified Sbp1 localisation (GFP) at 25°C. Merged images represent differential interference contrast (DIC, grey) filter showing the outline of the cell and green fluorescent protein channel (GFP, green) filter showing fluorescence. Magnification = 100x, scale bar = 10μm, represented by a white line. **B)** ImageJ quantification of mean fluorescent signal in the nucleus/cytoplasm. Statistical analysis (one-way ANOVA) showed a significant effect of strain on mean fluorescence ($p = 2.66 \times 10^{-13}$ ***), with Sbp1 protein increasing in all exosome mutants (p adjusted < 0.01, ***). **C)** Spot test of growth of tagged strains. Serial dilutions of *sbp1-GFP* (a), *sbp1-GFP dis3-54* (b), *sbp1-GFP rrp6Δ* (d), and *sbp1-GFP mtl1-1* (c), when compared to the untagged strains. Cells were spotted on YES and incubated at 25°C. Growth of tagged strains is comparable to wild-type.

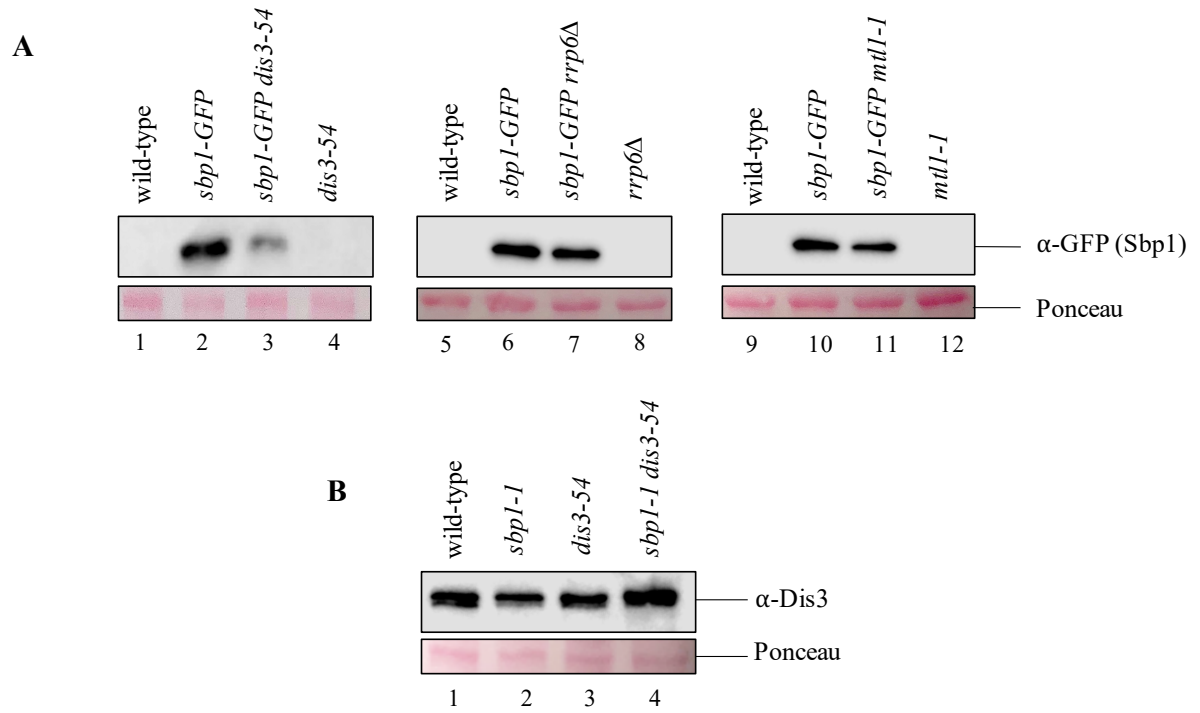


Figure 2.12: Sbp1 abundance is reduced in the *dis3-54* mutant. A) Sbp1 protein levels were assessed by western in wild-type, a *dis3-54* background (lanes 1-4), *rrp6Δ* (lanes 5-8), and *mtl1-1* (lanes 9-12) backgrounds. Proteins were separated on a 10% SDS-PAGE acrylamide gels and Sbp1 abundance was confirmed by antibody against GFP. Ponceau staining represents equal loading across all samples and strain backgrounds were included as no-tagged controls. Sbp1 protein abundance was shown to change in *dis3-54* alone. **B)** Dis3 protein levels in wild-type (lane 1), *sbp1-1* (lane 2), *dis3-54* (lane 3), and *sbp1-1 dis3-54* (lane 4) were assessed after a 4 hour shift to 37°C. Protein extracts were run on 8% SDS-PAGE acrylamide gels and Dis3 abundance was confirmed by an antibody against Dis3. Ponceau staining represents equal loading across all samples.

2.8 Discussion

Multiple RNAs have to be exported out of the nucleus in order for them to execute their function. Despite the fact that multiple links have been proposed between RNA export and decay, the question of how a cell deals with the decision to export or degrade RNA is not fully understood. Multiple studies have demonstrated that the accumulation of RNA in the nucleus, resulting from an impairment in either export or decay, can have a severe negative impact on the cell and can result in genome instability (Jimeno et al. 2002, Mikolaskova et al. 2018). Therefore, understanding the interplay between export and decay is central in unravelling how gene expression is regulated.

This chapter shows experimental evidence that does suggest a potential link between RNA degradation and export inside the nucleus. The data presented here demonstrate that a component of the fission yeast Ran cycle, the conserved protein Sbp1, plays a role in RNA export that aligns to the predicted role of Sbp1 as a RanBP in the Ran cycle (Cavazza & Vernos 2016). The main outstanding question is whether or not there is a direct connection between RNA export and decay.

The global nuclear RNA retention observed in this study is consistent with that observed in *S. cerevisiae* Ran-cycle ts mutants Prp20 (RanGEF) and Rna1 (RanGAP) (Forrester et al. 1992). This suggests that the RNA retention observed in this study is a common phenotype in Ran cycle mutants and that this is likely due to the role of the Ran cycle in nuclear export of RNA. Beyond the common phenotypes described here, there are other effects that seem to be found in mutants of proteins implicated in the Ran cycle and these effects are similar to exosome mutants. RNA processing defects have also been observed in Ran-cycle ts mutants, including in ts-Rcc1 and ts-Rna1 (Hopper et al. 1978, Kadowaki et al. 1993) and *S. cerevisiae* RanGTP has been shown to be important for RNA processing (Suzuki et al. 2001). RNA-processing defects need to be explored in *sbp1-1* to determine whether they can be observed when Sbp1 function is impaired.

Interestingly, many nuclear ncRNAs in *S. cerevisiae* have been suggested to

undergo shuttling between the nucleus and the cytoplasm (Becker et al. 2019, Wu et al. 2014), although this has not yet been identified in fission yeast. Considering this idea, nuclear retention in *dis3-54* could be explained by an impairment in Dis3-mediated degradation, while the RNA maintains the ability to shuttle between the nucleus and cytoplasm. Independently, in *sbp1-1* the ability to shuttle between the nucleus and cytoplasm might be impaired and therefore these transcripts remain nuclear localised. However, the impairment in degradation in *sbp1-1* could be explained by the Dis3-mediated degradation being dependent on the maturation in the cytoplasm prior to re-import. Further, this could explain the observed additive effect of an enhancement in nuclear RNA retention in the *sbp1-1 dis3-54* mutant, in which both degradation and export functions are impaired.

It remains to be explored whether or not the up-regulation of ncRNA observed in *sbp1-1* can be linked to the observed poly(A)⁺ retention, to identify whether the same up-regulated transcripts are normally exported via a mechanism that is dependent on the role of Sbp1. sm FISH with gene-specific probes for the up-regulated ncRNA transcripts identified from RNA-seq would give an insight into the localisation of these transcripts. Carrying this out in parallel in *dis3-54* with the same probes would help identify if these same up-regulated ncRNAs as in *sbp1-1* also contribute to the retention phenotype observed in *dis3-54* in this study. As discussed previously, the results presented here align with the possibility that a defect in Sbp1-mediated transport could partially explain the retention observed in *dis3-54*. This is suggested based on the observation of: 1) a reduction in Sbp1-Spi1 on poly(A)⁺ RNA in *dis3-54*, 2) a reduction in Sbp1 abundance in *dis3-54*, and 3) an alteration to the normal Sbp1 localisation in *dis3-54*. Over-expression of Sbp1 in a *dis3-54* background would test this hypothesis and would help identify whether the retention observed in *dis3-54* could be related to Sbp1 function.

Considering the importance of the Ran-binding domain for both the cellular localisation of Sbp1 (Novoa et al. 1999) and for binding to Ran (Vetter et al. 1999), it is plausible that the point mutation in the Ran-binding domain of *sbp1-1* could disrupt the normal cellular localisation of Sbp1 in *sbp1-1* or alter the interaction with the Spi1 Ran GTPase, either of which could impact the balance between the

active GTP-bound and the inactive GDP-bound form of Ran. Biochemical studies assessing the impact of *sbp1-1* on the ability of Sbp1 to interact with Spi1 are necessary in order to identify the direct impact on Ran activity and to confirm the assumption that any effect of Sbp1 occurs through Ran. Simply, Sbp1 localisation in *sbp1-1-GFP* and Ran GTPase binding assays in *sbp1-1* could be utilised to unravel the mechanistic impact of the Sbp1 point mutation.

In addition to the role of the Ran cycle in nucleocytoplasmic transport, Ran has also been shown to take part in other cellular processes. The Ran GTPase Spi1 has been implicated separately in cell cycle control, as a Spi1 mutant showed defects in chromosome segregation (Fleig et al. 2000). Interestingly, defects in chromosome segregation have also been reported for the Dis3 mutant (*dis3-54*) (Kinoshita et al. 1991), although the reasons behind this are less clear. Non-coding transcripts produced from the centromere, a region necessary for the segregation of chromosomes during mitosis, are suggested to be involved in the function of the centromere in many species (Cáceres-Gutiérrez & Herrera 2017). The exosome has been linked to the rapid turnover of ncRNAs produced from the centromeric regions (centromeric ncRNAs) (Wang et al. 2008, Choi et al. 2011) and accumulation of centromeric ncRNAs has been linked to defects in chromosome segregation (Cáceres-Gutiérrez & Herrera 2017). Likewise, the fission yeast export adaptor Mlo3 (homologue of mammalian ALYREF) has also been implicated in the silencing of centromeric transcripts (Reyes-Turcu et al. 2011, Zhang et al. 2011). Also, specific ncRNAs have been shown to be important for chromosome pairing (Ding et al. 2012, 2019, Yamashita, Kariyazono & Watanabe 2012). It is tempting to speculate that the accumulation of ncRNAs in the nucleus in exosome and export mutants could be a cause of the defective chromosome segregation reported previously. Nonetheless, further experiments are required to separate the implication of Sbp1 in mitotic control from the implication of Sbp1 in the exosome pathway. For example, mitotic defects could be assessed in the over-expressed Sbp1 strain in a *dis3-54* background as suggested previously.

It is also unclear how Sbp1 impacts nucleocytoplasmic transport. Identifying the mechanistic route employed by Sbp1-dependent RNA transport would further

support the suggested role of Sbp1 in nucleocytoplasmic transport. Although mRNA transport by Mex67 has been demonstrated in fission yeast, it is less clear if the role of Crm1 in ncRNA transport is conserved. Ran has been linked to karyopherin-mediated import and export in mammals (Kohler & Hurt 2007). This thesis showed that Sbp1 interacts with an importin of the karyopherin family, implying that Ran-mediated karyopherin import is conserved in fission yeast. However, whether or not the karyopherin-family export receptors are also utilised for Ran-mediated export remains to be clarified. Identifying the subtype of ncRNA that constitute the export defect in *sbp1-1* would also help identify the export receptor utilised by Sbp1 for RNA export and would further support the role of Sbp1 in nucleocytoplasmic transport. For instance, if the up-regulated ncRNAs are confirmed to be nuclear localised by sm FISH as discussed previously, this same experiment could be repeated for several distinct export receptor mutants. It could be suggested that if a phenotype of nuclear gene-specific up-regulation is mimicked in nuclear transport receptor mutants then this subtype is likely to depend on the same nuclear transport receptor for export. For instance, sm FISH with gene-specific probes using *sbp1-1*, and mutants of Crm1 and Mex67-Mtr2 will help identify the nuclear transport receptor utilised for Sbp1-mediated nucleocytoplasmic export.

In summary, these data identified Sbp1 as a Ran GTPase-related protein that may be linked to the nuclear RNA exosome, due to similar phenotypes being observed between a mutant of Sbp1 and Dis3. Additionally, Sbp1 and the nuclear RNA exosome seem to regulate the expression of a common subset of genes and Sbp1 abundance was reduced in a *dis3-54* mutant. Identifying the RNAs that contribute to the export defect observed upon impairment of Sbp1 function is crucial in understanding the role of Sbp1 and further clarifying the link between the exosome and Sbp1.

Chapter 3

Exploring the possible impact of the altered RNA binding of the exosome observed in exosome mutants on the integrity of the exosome complex

3.1 Introduction

As discussed previously, prior work in the lab has identified that the ability of Mtl1 to cross-link to RNA is lost in *mtl1-1*, suggesting that the exosome binding to RNA is reduced in this mutant (Kilchert et al. BioRxiv, 2019, accepted to Genome Research 2020) (Figure 1.5B). The reduction in RNA cross-linking observed in the *mtl1-1* mutant suggests that the area surrounding the arch domain of Mtl1/Mtr4 where these mutations are found (Figure 1.5A) is important for RNA interaction of this conserved helicase. Further, many RNA-binding proteins involved in substrate recognition are increased in *mtl1-1*, in contrast to components of the exosome and Mtl1 (Figure 1.5).

This chapter builds on the data generated from the RNA-interactome capture to build on the idea of a two-step mechanism responsible for substrate recognition by the exosome.

3.2 Mtl1 protein abundance is not altered in *mtl1-1*

To test the idea that the observed differences in exosome behaviour in *mtl1-1* (Figure 1.5) were due to an alteration in Mtl1 protein abundance, the levels of tagged Mtl1 were assessed. To do this, proteins were extracted from Flag tagged Mtl1 in a WT background (*mtl1-Flag*), Flag tagged Mtl1 in an *mtl1-1* background (*mtl1-1-Flag*), and an untagged control. Proteins extracted from these strains were separated by SDS-PAGE electrophoresis and Mtl1 abundance was assessed with an antibody against Flag (Table 5.4). An antibody against Tubulin was used as a loading control (Table 5.4) to exclude the possibility that any alterations in Mtl1 abundance were due to discrepancies in loading. However, Mtl1 showed similar abundance in protein levels in *mtl1-1* to those observed in the WT background (Figure 3.1, compare lane 2 with lane 3). Interestingly, this analysis of Mtl1 levels revealed Mtl1 migrating slower on the SDS-PAGE gel in *mtl1-1-Flag* compared to *mtl1-Flag*, possibly indicating that protein is post-translationally modified in this mutant (Figure 3.1, compare lane 3 to lane 2). Together, these data suggest that Mtl1 protein abundance is unaltered by the partial loss of function mutant *mtl1-1*.

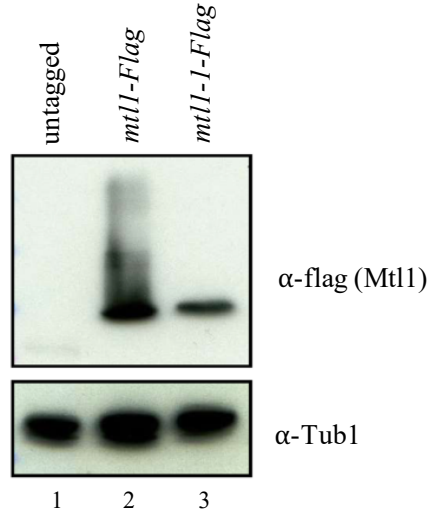


Figure 3.1: **Mtl1 protein levels are similar between *mtl1-1* and wild-type.** *S. pombe* lysate from an untagged strain (lane 1), *mtl1-Flag* (lane 2), or *mtl1-1-Flag* (lane 3) strains at 30°C. Lysate was loaded onto SDS-PAGE and proteins were analysed by western blot with an antibody against Flag (Mtl1) for detection of Mtl1 and an antibody against Tubulin-1 (Tub1) for a loading control.

3.3 Exosome integrity is preserved in *mtl1-1*

Since Mtl1 protein abundance was not altered when Mtl1 was mutated (Figure 3.1), it was speculated that the difference in the occupancy of the exosome on RNA in *mtl1-1* may be due to compromised exosome integrity in this mutant. Therefore, the next line of research was targeted towards testing this question by purifying *S. pombe* native exosome complexes from the *mtl1-1* mutant to analyse whether the exosome complex retains all subunits in this background. To do this, the exonuclease Rrp6 was endogenously tagged with a Strep tag in both WT and *mtl1-1* backgrounds (Figure 3.2A). Strep was chosen as Mtl1-1 was already endogenously tagged with a C-terminal Flag tag (Lee et al. 2013). The Strep tag was used to pull down the Rrp6 exosome through affinity purification. Rrp6 associated exosome complexes were then further isolated from proteins not associated with the exosome complex by size exclusion chromatography (Figure 3.2A). Fractions corresponding to the exosome as identified by Rrp6-Strep were isolated and analysed by SDS-PAGE and followed by coomassie staining (Figure 3.2A). As a control, Rrp6 exosome was also

purified from WT and a *dis3-54* background (Figure 3.2B).

The coomassie staining showed a similar profile of proteins when the Rrp6 exosome was purified from WT and *mtl1-1* (Figure 3.2A, compare lanes 1 and 2) and when the Rrp6 exosome was purified from WT and *dis3-54* (Figure 3.2B, compare lanes 1 and 2) and western blot showed that Dis3 was present in all backgrounds (Figure 3.2). MS analysis of size exclusion chromatography from the Dis3 purification set (Figure 3.2B gel) confirmed the identity of the exosome subunits (Table 3.1). Taken together, these data suggest that the integrity of the Rrp6 exosome is maintained in both *mtl1-1* and *dis3-54* and suggests that all exosome subunits co-purify as a complex in both of these exosome mutant backgrounds.

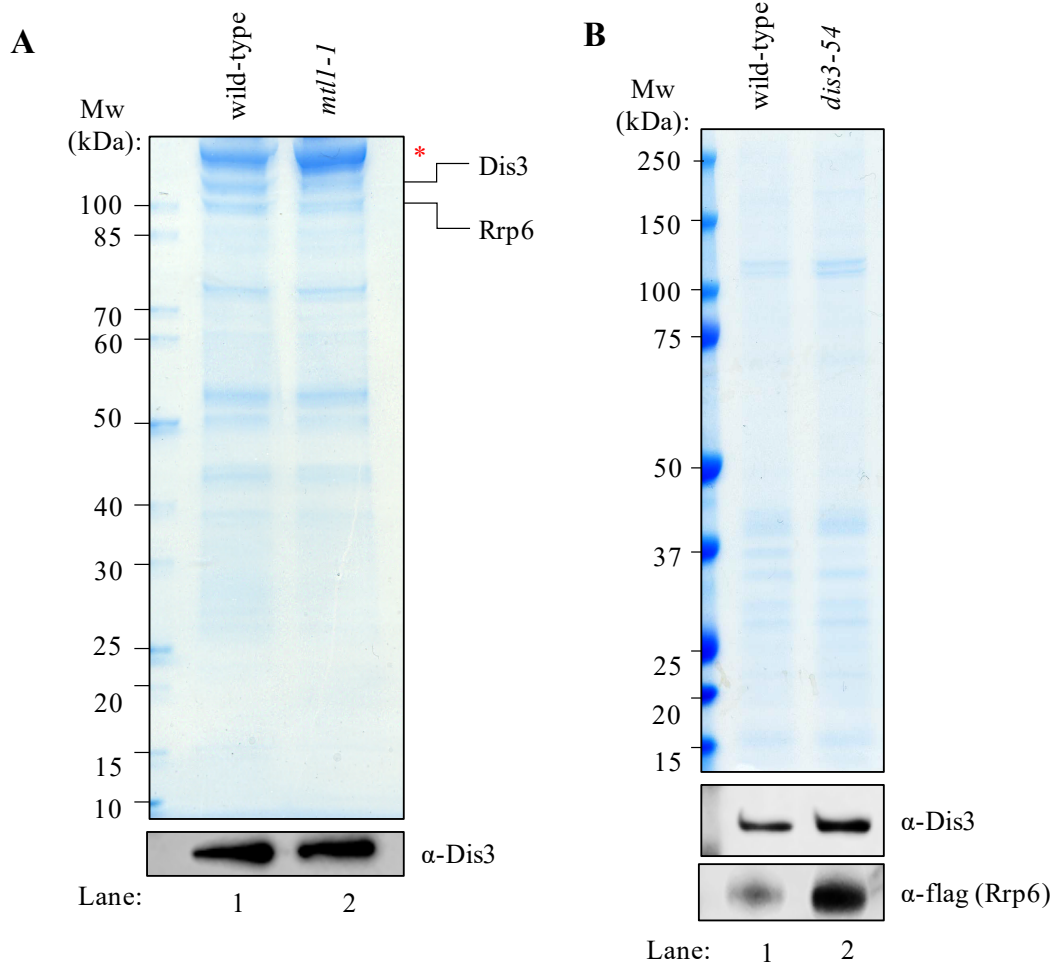


Figure 3.2: **Exosome integrity in exosome mutants *mtl1-1* and *dis3-54*.** Exosome integrity was assessed by endogenous tagging of **A)** Rrp6-Strep in wild-type (lane 1) and Rrp6-Strep in *mtl1-1* (lane 2). **B)** Rrp6-3HA-3Flag in wild-type (lane 1) and Rrp6-3HA-3Flag in *dis3-54* (lane 2). Both sets were subjected to affinity purification using their respective tags and size exclusion chromatography. Proteins were subjected to SDS-gel electrophoresis on a NuPAGE Bis-Tris precast gel and visualised by coomassie staining. The band corresponding to non-specific protein co-purified with the *S. pombe* native exosome complex via Strep column is indicated with a star (*). Dis3 presence was assessed by western blot against Dis3. Rrp6 presence was assessed by western blot against Flag.

Table 3.1: **Mass-spectrometry results of Rrp6 two step purification.** Components of the exosome are highlighted according to the structure of the exosome in Figure 1.1: PH-ring proteins (blue), cap proteins (green), adaptor proteins (grey) and nucleases (red, purple). The values indicate the intensity score of protein identified in wild-type fractions or in *dis3-54* fractions.

Exosome component	Intensity score	
	wild-type	<i>dis3-54</i>
Rrp41	1.10E+05	-
Rrp4	1.70E+07	7.10E+06
Rrp46	2.00E+07	9.60E+06
Rrp43	2.80E+07	3.40E+06
Rrp42	2.90E+07	6.60E+06
Csl4	3.10E+07	1.80E+07
Mtr3	3.60E+07	1.60E+07
Rrp40	8.30E+07	2.60E+07
Dis3	1.40E+08	7.80E+07
Rrp45	1.60E+08	9.50E+07
Rrp6	7.50E+08	3.90E+08
Mpp6	-	-

3.4 Discussion

Studying differences between wild-type exosome-related proteins and exosome-mutants can provide an insight into the structure of the exosome. In this study, native affinity purification was employed to identify which components of the exosome co-purify as a complex and whether the composition of this complex differs in exosome mutant backgrounds. The data presented here show that the observed reduction in the cross-linking of the exosome to RNA in *mtl1-1* (Kilchert et al. BioRxiv, 2019, accepted to Genome Research 2020) is not due to either alterations in protein abundance of Mtl1 or alterations in the ability of the core exosome to associate with the nucleases that provide catalytic activity (Dis3 and Rrp6).

The RNA nuclear exosome and co-factors involved in exosome-targeting (e.g.

Mmi1) have been suggested to be post-translationally modified (Telekawa et al. 2018). Also, many proteins associated with exosome function are found to be phosphorylated, including Red1, Red5, and Iss10 (Lock et al. 2018), although the significance of these modifications remains elusive. It is possible that the altered migration of Mtl1 is due to alterations in post-translational modifications in *mtl1-1*. Nonetheless, it would be interesting to assess the post-translational modifications of exosome-related co-factors in the future and to test their possible functional contribution to exosome regulation.

Previous studies have shown that the helicases Mtr4/Mtl1 can contact both the exosome and RNA-binding proteins that are suggested to be important for exosome regulation. For instance, human Mtr4 (hMTR4) has been shown to associate with the exosome core (Weick et al. 2018, Lubas et al. 2011) and affinity purification has identified interaction partners of Mtl1 to include many other RNA-binding proteins (Egan et al. 2014, Lee et al. 2013, Zhou et al. 2015). Therefore, Mtl1 might play a role in coupling RNA recognition to exosome targeting. Further, RNA-binding proteins important for substrate recognition (e.g. Mmi1) and other exosome-related proteins (e.g. Red1) were increased or unaltered on RNA despite the altered exosome occupancy on poly(A)+ RNA in *mtl1-1* (Birot et al. unpublished manuscript from the Vasilieva lab, 2020), suggesting the recruitment of these factors to RNA are independent of the exosome and of Mtl1. The results presented here suggest that exosome integrity is retained in *mtl1-1* as the exosome co-purifies as a complex. This aligns with the model of a two-step mechanism for exosome recruitment to substrates, in which Mtl1 contacts substrates and results in the recruitment of the exosome to substrates (Birot et al. unpublished manuscript from the Vasilieva lab, 2020). However, MS needs to be carried out in the purification of Rrp6 from the *mtl1-1* background to confirm the presence of exosome components. Nonetheless, these results are consistent with the idea that a specific ribonucleoparticle is assembled and then targeted by the nuclear exosome through the specific helicase Mtl1 in fission yeast.

Chapter 4

General discussion and conclusions

The work presented here demonstrates how results from a proteomic poly(A)+ pull down method can be explored to understand more about the function and regulation of the RNA exosome. First, I focused on the fission yeast export-related protein Sbp1, which was identified to have a reduced occupancy on poly(A)+ RNA in the pull down of proteins bound to poly(A)+ RNA from the *dis3-54* mutant. Next, I used native affinity purification of the exosome in order to assess the integrity of the exosome as a complex in the *mtl1-1* mutant.

4.1 Exploring the role of the ‘RNA-interactome’ candidate protein Sbp1

4.1.1 Does the role of Sbp1 align with the predicted role from other organisms?

Here, the fission yeast RanBP Sbp1 (orthologue of mammalian RanBP1) was studied to investigate a potential link between RNA export and the RNA exosome complex. First, functional analysis of Sbp1 was carried out to assess whether the role of the protein aligns to that of other RanBPs. Second, a temperature sensitive mutant of Sbp1 was created to help explore the function of Sbp1 in fission yeast. I show

that Sbp1 plays a role in the fission yeast Ran cycle, since the wild-type protein was shown to interact with other characterised members of the fission yeast Ran cycle. Next, I show that a temperature sensitive mutant of Sbp1 has an export defect at its restrictive temperature, linking the role of Sbp1 in the Ran cycle with a role in RNA transport.

Although Sbp1 is implicated in export, the specific species of RNA targeted by Sbp1 is less clear. I provide evidence to suggest that several ncRNAs are targeted by Sbp1, although further experiments are necessary to determine whether other phenotypes also observed in RNA biogenesis in *sbp1-1* can be explained by the same ncRNAs. Studies in mammals show that Ran is mainly utilised for Crm1 karyopherin export. Additional work is required in order to show that Ran utilises Crm1 for export, which would further support the suggestion that Sbp1 is important for ncRNA transport and the predicted role of the Ran cycle in Crm1-mediated nucleocytoplasmic export.

4.1.2 Can Sbp1 be linked to the exosome?

The precise association between the Ran cycle and the exosome still remains an open question. I provide additional evidence to further support the suggestion that any role that Sbp1 holds in exosome regulation is most likely due to an interaction with the Dis3 exosome, either directly or indirectly. Similarly, I show that impairing either Dis3 or Sbp1 function results in the up-regulation of many transcripts encoding for ncRNAs. Additionally, I demonstrate nuclear retention of poly(A)⁺ RNA in a *dis3-54* background, further strengthening the suggestion that Sbp1 and Dis3 are also connected on the transcript level. Finally, I show that a temperature sensitive mutant of Sbp1 has a synthetic lethality defect when combined with exosome subunits.

I provide evidence to suggest that the retention observed in *dis3-54* could be in part due to a disruption to the role of Sbp1 in the Ran cycle and subsequent RNA export for several reasons. Firstly, nuclear retention of poly(A)⁺ RNA is a common

phenotype in both Dis3 and Sbp1 mutants. Secondly, disruptions to the normal localisation of Sbp1 is observed in *dis3-54*. Lastly, Sbp1 protein abundance is shown to be reduced in *dis3-54*. However, further work is required to confirm the link between Dis3 and either Sbp1 or Sbp1-Spi1. I also suggest that these results could be in part described by the regulation of nucleocytoplasmic shuttling of ncRNAs by Sbp1, although this has yet to be identified in fission yeast.

4.2 Exploring the RNA exosome integrity

This data presented here builds on the observation by Kilchert et al. (BioRxiv, 2019, accepted to Genome Research 2020) and Birot et al. (unpublished manuscript from the Vasilieva lab, 2020) of a reduction in RNA-exosome binding in a *mtl1-1* background. I show that native affinity purification in fission yeast can demonstrate that the exosome co-immunoprecipitates as a complex in both *mtl1-1* and *dis3-54*.

Therefore, I present data to support the idea of a two-step mechanism of substrate recognition: 1) recognition of target RNA by co-factors, 2) recruitment of the exosome to RNA. Structural work of the composition of the exosome in the catalytic mutants would be the gold standard in proving that the exosome integrity is maintained in catalytic mutants and therefore would support this idea of two-step regulation of the exosome.

Chapter 5

Materials and methods

Table 5.1: Oligonucleotides used in this study

Name	Sequence	Description
Sbp1 Sal I Fw	TCAGATTCGTCGACATGTCCGCAGAACAA- GAAAA	<i>sbp1-1</i> cloning
Sbp1BgIII Rev	TCAGACTTAGATCTCTTACATA- GACGTTTCATTGA	<i>sbp1-1</i> cloning
Sbp1 Fw	ATGTCCGCAGAACAAAGAAAA	<i>sbp1-1</i> cloning
Sbp1 Rev	ATAATAACAATATGCATTTGCTCCATG- CACCAGCTAACTTTACCTTTGTACGATA- CATTTTCATTCTACAATGGTTAAACAGTATA GCGACCAGCA	<i>sbp1-1</i> cloning
Sbp1 upstream fw	GCTTTAAGGCAAAGATTTTATTAGCA	Sbp1 screening
Sbp1 downstream rev	CAGATCCGTATACCATTAACCA	Sbp1 screening
Sbp1 rev	CCACCCTAAAAGAACTGCAAA	Sbp1 screening

Continued on next page

Name	Sequence	Description
Sbp1 Flag fw	AAACCTCGTTAGATGCCAATCTCTT- TAAGGAAAACCTTTGAAAAAT- ACCAAGAAGAGAAT- GCCAAAATTCTCAAAAAAATGACTAC AAAGACCATGACGG	Sbp1 tagging
Sbp1 Flag rev	ATCAACTTTACCGTGAGAAG- GAAAAAGATGGAAAAAAGT- TAATTCTATTATAGCAAGC- CCAGAATAAATGACGATATAAATGGATGG CGGCGTTAGTATC	Sbp1 tagging
Sbp1 GFP fw	AAACCTCGTTAGATGCCAATCTCTT- TAAGGAAAACCTTTGAAAAAT- ACCAAGAAGAGAAT- GCCAAAATTCTCAAAAAAATAGTAAA GGAGAAGAACTTTT	Sbp1 tagging
Sbp1 GFP rev	ATCAACTTTACCGTGAGAAG- GAAAAAGATGGAAAAAAGT- TAATTCTATTATAGCAAGC- CCAGAATAAATGACGATATAAACAGTATAG CGACCAGCATTC	Sbp1 tagging
ncRNA.1114 fw	CGATTTTACCCAACATGTGC	Northern probe
ncRNA.1114 rev	TCCATAACATTCATCCCATCTTC	Northern probe
Sme2 fw	TGCATGCAAGATCGCTTTAG	Northern probe
Sme2 rev	ACCGAATCCAGCTTTTTTGAA	Northern probe
Rrp6 5'UTR	TTGATTGTTTCATCCATTCTAGATTATCC	Confirming double mutants

Continued on next page

Name	Sequence	Description
Rrp6 3'UTR	AGAGGAATTATTAAATCACCCGTTTATG	Confirming double mutants/Rrp6 tagging
Dis3 fw	TGGAGAAATGGAAACCAAGG	Confirming double mutants
Dis3 rev	TCATCAATTCGAGCTTGTGC	Confirming double mutants
Mtl1 fw	ATGCGTTCGCTTTTACTTCG	Confirming double mutants
Mtl1 rev	TTTGATAGGCCAAACGATCA	Confirming double mutants
Rrp6-L5	AAACGAGCTCGAATTCATCGAT- GATATATCAGTTTTAATTTTAAATAAAC- GAAACA	Rrp6 tagging
Rrp6-C term	AATGTAAAGCAGTCCACTGTAAGTGAAG	Rrp6 tagging
Rrp6-L4	TAATTAACCCGGGGATCCGTCGACCATAA- GATGTACTCTTCCCGTCTGAAACCTT	Rrp6 tagging
Strep fw	TCGACGGATCCCCGGGTTAATTAACGAG- GATCTGTACTTTCAGAGCTG	Rrp6 tagging
Strep rev	GGCTGCAGGAATTCGATATCAAGCTT- TATTTCTCAAAGTGAAGATGGCTCCAAC- CTGCACCGCTAC	Rrp6 tagging
Nat Vector fw	AGCTTGATATCGAATTCCTGCAG	Rrp6 tagging
Nat Vector rev	GTTAATTAACCCGGGGATCC	Rrp6 tagging

Table 5.3: Plasmids used in this study

Number	Description	Used for	Source
4026	pFA6a 3xHA, 3xFlag with 6xGly linker, KanMX6	Amplification of Flag Kan for endogenous tagging	Vasilieva Lab
4027	pAG25 3xHA, 3xFlag with 6xGly linker ,terminator, natMX4	Amplification of Flag Nat for endogenous tagging	Vasilieva Lab
3596	pFA6a-HA-GFP-NatMX	Amplification of GFP tag for endogenous tagging	Vasilieva Lab
3972	3xHA, strepII, KanMX6, AmpR	Amplification of Strep tag for endogenous tagging	Vasilieva Lab

Table 5.4: Antibodies used in this study

Antibody	Origin	Source	Purpose
α -Dis3	Rabbit	Gentaur	Western blot (1:2000)
α -GFP	Rabbit	Abcam	Western blot (1:2500)
α -Strep-Tactin-HRP	Rabbit	IBA	Western blot (1:4000)
α -Flag-M2-HRP	Mouse	Sigma	Western blot (1:5000)
α -Rabbit	Goat	Sigma	Western blot (1:10000)

Table 5.5: Strains used in this study

Number	Genotype	Description	Constructed by
YP67	h- <i>dis3-54 leu1-32 ura4-Δ18</i>	<i>dis3-54</i>	Kinoshita et al. (1991)
YP71	h-	Wild-type	Vasilieva Lab
YP144	h+ <i>ura4-Δ18 leu1-32 his3Δ::1 ade6-M216</i>	Wild-type	Lemieux et al. (2011)
YP729	h+ <i>mtl1-1::Flag::kanMX6 ura4-Δ18 leu1-32 his3Δ::1 ade6-M216</i>	<i>mtl1-1</i>	Lee et al. (2013)
YP775	h- <i>mtl1::Flag::kanMX6 ura4-Δ18 leu1-32 his3Δ::1 ade6-M216</i>	Mtl1-Flag	Lee et al. (2013)
YP1001	h+ <i>rrp6::natMX6, leu1-32, ura4-Δ18</i>	<i>rrp6Δ</i> for Sbp1 cross	Vasilieva Lab
YP1004	h- <i>rrp6::natMX6, leu1-32</i>	<i>rrp6Δ</i> for FISH	Vasilieva Lab
YP1057	h+ <i>dis3-54, leu1-32</i>	<i>dis3-54</i> for Sbp1 cross	Kinoshita et al. (1991)
YP1416	h- <i>sbp1-1::kanMX6</i>	<i>sbp1-1</i>	This study
YP1438	h- <i>sbp1-1::kanMX6, dis3-54</i>	<i>sbp1-1, dis3-54</i>	This study
YP1468	h+ <i>mCherry-mtl1::hygMX6</i>	Mtl1 mCherry localisation	Adrien Birot, Vasilieva Lab
YP1471	h+ <i>mCherry-rrp6::hygMX6</i>	Rrp6 mCherry localisation	Adrien Birot, Vasilieva Lab
YP1476	h- <i>sbp1-1::kanMX6, rrp6::natMX6</i>	<i>sbp1-1, rrp6Δ</i>	This study
YP1494	h- <i>sbp1-GFP::kanMX6</i>	Sbp1-GFP localisation	This study
YP1496	h- <i>sbp1-Flag::natMX6</i>	Sbp1-Flag	This study

Continued on next page

Number	Genotype	Description	Constructed by
YP1541	h- <i>sbp1-GFP::kanMX6</i> , <i>dis3-54</i>	Sbp1-GFP localisation in <i>dis3-54</i>	This study
YP1543	h- <i>sbp1-GFP::kanMX6</i> <i>rrp6Δ</i>	Sbp1-GFP localisation in <i>rrp6Δ</i>	This study
YP1543	h- <i>sbp1-GFP::kanMX6</i> , <i>mtl1-1</i>	Sbp1-GFP localisation in <i>mtl1-1</i>	This study
YP1544	h- <i>sbp1-GFP::kanMX6</i> , <i>mtl1-1</i>	Sbp1-GFP localisation in <i>mtl1-1</i>	This study
YP1546	h- <i>mCherry-rrp6::hygMX6</i> , <i>sbp1-1::kanMX6</i>	Rrp6 mCherry localisation in <i>sbp1-1</i>	This study
YP1547	h- <i>mCherry-mtl1-1::hygMX6</i> , <i>sbp1-1::kanMX6</i>	mtl1-1 mCherry localisation in <i>sbp1-1</i>	This study
YP1622	h? <i>rrp6-3HA-3Flag::kanMX6</i> <i>ura4-Δ18</i> <i>leu1-32 his3Δ::1 ade6-M216</i>	Rrp6 flag in wild-type	Dr. Cornelia Kilchert, Giessen university
YP1681	h? <i>rrp6-3HA-3Flag::kanMX6</i> <i>dis3-54</i> <i>ura4-Δ18 leu1-32 his3Δ::1 ade6-M216</i>	Rrp6 flag in <i>dis3-54</i>	Dr. Cornelia Kilchert, Giessen university
YP1833	h+ <i>rrp6-Strep::natMX6</i> <i>ura4-Δ18 leu1-32 his3Δ::1 ade6-M216</i>	Rrp6 strep in wild-type	This study
YP1834	h+ <i>rrp6-Strep::natMX6</i> <i>mtl1-1::Flag::kanMX6</i> <i>ura4-Δ18 leu1-32 his3Δ::1 ade6-M216</i>	Rrp6 strep in <i>mtl1-1</i>	This study

Continued on next page

Number	Genotype	Description	Constructed by
YF336	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	<i>S. cerevisiae</i> for RNA-seq spike in	Vasilieva Lab

5.1 Strain construction and growth

5.1.1 *S. pombe* growth conditions

S. pombe was grown in YES liquid media (0.5% yeast extract, 3% glucose, 2% bacto-agar, 150mg/L adenine, 100mg/L uracil, 100mg/L histidine, 100mg/L leucine) at the temperatures indicated.

5.1.2 Creation of strains

Strains of an opposite mating type were crossed on EMMG (14.7mM potassium hydrogen phthalate, 15.5mM Na₂HPO₄, 93.5mM NH₄Cl, 2% w/v glucose, 20ml/L salt stock, 1ml/L vitamin stock, 0.1ml/L mineral stock). When tetrads were visible by light microscope (around 3-4 days later), Random-Spore Analysis (RSA) was performed. For each cross, 300 spores were plated on EMMG plates and left to grow for 3-6 days depending on the temperature of incubation. Replica plating was carried out to select the appropriate genotype. Temperature sensitive mutations were screened for by replica plating onto YES+phloxine and incubation at 18°C, 30°C, and 37°C.

5.1.3 Transformation

For all tagged strains, DNA was amplified by PCR as described previously and the resulting product was integrated by homologous recombination using transformation in a background strain (Table 5.5). Cells were grown in YES overnight to an optical density measured at 600nm (OD₆₀₀) of 0.7. Cells were spun down at 3000rpm for 2 minutes and washed twice with 1ml of LiAc buffer (0.1M Li-acetate pH 4.9). Cells

were re-suspended in 150 μ l of LiAc buffer and incubated for 45 minutes at 30°C (non-shaker). 15 μ l of DNA was added (not more than 2 μ g) and mixed by vortexing. A volume (290 μ l per transformation) of 50% w/v PEG (pH 7.5, pre-warmed at 30°C) was added to the solution and incubated at 30°C for 60 minutes. Cells were heat-shocked for 15 minutes in a 45°C water-bath, followed by 10 minutes at room temperature. Samples were washed with YES to remove any residual PEG and cultured in 5ml total volume of YES overnight. Then cells were washed with YES and resuspended in 1ml of YES and spread onto YES+ antibiotic plates and incubated at 30°C. After roughly 5 days, transformants were selected and the genotype was confirmed by replica plating of YES plates onto EMMG – auxotrophic markers. Temperature sensitive mutations were screened for by replica plating onto phloxine and incubation at 18°C, 30°C, and 37°C. All transformants were confirmed by colony-PCR and DNA sequencing. Tagged strains were confirmed by colony PCR, western blot, and by microscopy where possible (GFP/mCherry).

5.1.4 Spot test

Strains were grown in YES at 30°C until 1×10^7 cells/ml the following morning. Cells were centrifuged at 3000rpm for 2 minutes, washed with YES, and diluted to 0.1 OD₆₀₀ in 1ml 1.5×10^6 . Five serial dilutions (1:5, 200 μ l) were created from the 1ml of 0.1 OD₆₀₀ and 5 μ l of each strain plus dilutions was spotted onto one YES plate and one phloxine plate. Two plates (one YES, one phloxine) were incubated at each temperature: 18°C, 25°C, 34°C, and 37°C for 6 days. Plates were scanned on different days to create the resulting image.

5.2 Molecular biology techniques

5.2.1 Polymerase Chain Reaction (PCR)

PCR mixtures were carried out in a volume of 50 μ l (0.5 μ M forward primer (Table 5.1), 0.5 μ M reverse primer (Table 5.1), 200 μ M dNTP, 1x polymerase buffer, 3% DMSO, and 1 μ l of Phire II (Thermofisher) or Phusion (NEB)). Template DNA was added to a concentration of 50ng (wild-type gDNA) or 1ng (plasmid DNA, Table 5.3) per 50 μ l PCR reaction. DNA was then subjected to the following steps:

1. Initial denaturation (98°C, 2 minutes)
2. Denaturation (98°C, 30 seconds)
3. Annealing (58°C, 30 seconds)
4. Extension (72°C, 15s/kb for Phire and 30s/kb for Phusion)
5. Final extension (72°C, 2 minutes)
6. Hold (4°C)

Steps 2-4 were repeated for 34 cycles. The same protocol was applied to colony PCR but with 10 minutes initial denaturation. Amplified DNA was separated on an 0.8-1.2% agarose gel with EtBr (0.05 g/mL) and visualised with a GelDoc system (Bio-rad).

5.2.2 Error-prone PCR mutagenesis

Primers were designed (Thermofisher, Table 5.1) to amplify from the start of Sbp1 to the end of the 3'UTR. Primers were also designed to amplify kanamycin from a plasmid (pFa6a-KanMX6, Table 5.3) to be homologous to the Sbp1 gene amplified. Quick ligase (NEB) was used to ligate the overhangs between the vector and Sbp1. The resulting vector was transformed into competent cells (TOP10 *E. coli*).

A thermocycler was used to introduce random point mutations into the vector containing the Sbp1 gene using a low-fidelity polymerase Phire (Thermofisher). In addition to the normal PCR reagents (1x Phire reaction buffer (Thermofisher), 0.5 μ M forward primer (Table 5.1), 0.5 μ M reverse primer (Table 5.1)), three different PCR conditions were used to create an unfavourable reaction:

1. 0.2mM dATP, 0.2mM dGTP, 1mM dCTP, 1mM dTTP
2. 7mM MgCl₂, 0.2mM dNTP
3. 0.2mM dCTP, 0.2mM dTTP, 1mM dATP, 1mM dGTP

The mutagenised DNA product was transformed into *S. pombe* wild-type strain (Table 5.5) as previously described, to create YP1416. Transformants were screened for temperature sensitivity by replica plating on YES+ phloxine at 37°C and 30°C.

5.2.3 Fluorescent in-situ hybridisation (FISH)

Strains were grown overnight at 30°C to log-phase $OD_{600} = 0.7$, 1×10^7 in 50 ml of Yes. Cultures were split in half, with 25ml of culture remaining at 25°C and 25ml of culture being shifted to 37°C. 4 hours later, 20ml of culture from both temperatures was fixed using 3.7% formaldehyde (2ml of 37% formaldehyde (Fisher) for 20ml of culture) for 30 minutes. Cells were washed twice with 1ml of ice-cold buffer B (1.2M sorbitol, 100mM $KHPO_4$ at pH 7.5) and resuspended in 1ml of spheroblasting buffer (890 μ l buffer B, 100 μ l of 200mM RVC, 10 μ l of 50mg/ml zymolase, 2 μ l mercaptoethanol). Cells were incubated in spheroblasting buffer at 37°C for 1 hour, or until 80% of cells became translucent on the microscope. Spheroblasting was stopped by washing of cells with 1ml of cold buffer B and incubated for 20 minutes at room temperature in 0.01% triton/1xPBS. After washing twice with 10% formamide/2xSSC, cells were divided into two tubes. RNase (100 μ g/ml final concentration) was added to one tube and both tubes were incubated at 37°C for 1 hour. Probe solution was made: 25ng of Alexa488-conjugated oligo(dT)₂₀ probe (Invitrogen), 10mg/ml tRNA, 5mg/ml ssDNA. Probe solution was dried in a vacuum concentrator and resuspended in hybridization buffer (20% formamide, 10mM $NaHPO_4$, 3 minutes at 95°C). After incubation, solutions were washed with 10% formamide/2xSSC and resuspended in 50 μ l of probe-hybridization buffer, and 50 μ l of buffer H (4xSSC, 4mg/ml BSA, 20mM RVC) and incubated overnight at 37°C at 200rpm. The following morning, cells were washed 3 times (10% formamide/2xSSC, 0.1% triton/2xSSC, 1xPBS), stained with DAPI (5mg/ml) and washed with 1xPBS. The final washed sample was resuspended in 100 μ l of 1xPBS and 5 μ l of sample was mounted onto a slide and covered with a cover slip. Samples were viewed on the spinning-disk microscope and Z-stack images were taken at 100x magnification.

5.2.4 Live microscopy

Cells were grown overnight in 5ml of YES at 30°C. 10 μ l of the sample was placed on an agarose pad immobilised on a microscope slide and immediately covered with a cover slip. Images were taken on a spinning-disk microscope set to 25°C at 100x magnification using the GFP and DIC filter.

5.2.5 Fixed microscopy

Cells were grown overnight in 50ml of YES at 25°C to 6×10^6 cells/ml. Then 25ml was kept at 25°C and 25ml was shifted to 37°C in a water tank. After 4 hours, both cultures at 25°C and 37°C were spun down and resuspended by vortexing with 1ml 70% cold ethanol and stored at -20°C. Cells were rehydrated by washing with water and resuspension in 1ml of water. Cells were stained with DAPI (5mg/ml) in 1xPBS. After 10 minutes in the dark, cells were spun down and resuspended in 1xPBS. 10 μ l of sample was mounted on a slide and covered with a cover slip. Samples were viewed on a spinning-disk microscope at 63x magnification using the mCherry filter and the DAPI filter.

5.3 Biochemical techniques

5.3.1 RNA extraction

An overnight separate culture of *S. pombe* and *S. cerevisiae* was inoculated in 100ml of YES at 25°C and YP + glucose (20%) respectively. Then, 50ml of cultures were split to 37°C and 50ml remained at 25°C. A similar amount of *S. cerevisiae* cells were split in a different culture to allow a spike-in normalisation process (Hu et al. 2015) used later. After 4 hours, cells were spun down and the pellet was re-suspended in 1ml dH₂O and transferred to 1.5ml eppendorph tubes. Cells were re-suspended in 500 μ l cold AE buffer (50mM NaAc pH5.0, 10mM EDTA, pH 8.0)

by vortexing. SDS (1%) and an equal amount of phenol-chloroform was added. Samples were vortexed for 20 seconds at max speed and transferred to 65°C for 5 minutes. Tubes were transferred to an ice bath and incubated for 10 minutes, followed by micro centrifugation for 15 minutes at 14000rpm at 4°C. The aqueous phase was transferred to a new tube and an equal amount of phenol-chloroform was added. Samples were re-centrifuged (15 minutes 14000rpm 4°C). The aqueous phase was added to a new tube and 1ml of EtOH (67% final) and 3M NaAc (2.6% final, pH 5.3-6.5), inverted 5 times to mix and precipitated at -20°C for 1 hour. Samples were micro centrifuged for 30 minutes (25000g, 4°C) and washed with 1ml of 70% EtOH. After micro centrifugation, pellet was air dried and dissolved in 100µl of dH₂O. Nanodrop was used to quantify the concentration of RNA and RNA integrity was confirmed by running a 1% agarose gel. RNA was stored at -20°C overnight. A biological replicate was carried out.

5.3.2 Protein extraction

S. pombe strains were cultured as previously described in RNA extraction, overnight at 25°C and then harvested or with a 4 hour shift to 37°C where stated. 2×10^8 cells were spun down (3500rcf, 2 minutes, 4°C) and washed twice with dH₂O and stored on dry ice. The cell pellet was dissolved in 125µl of cold lysis buffer (50mM Tris pH7.5, 150mM NaCl, 1mM MgCl₂, 1mM DTT, 0.5% Triton X-100, Glycerol 10%, 1mM PMSF, 1x pierce protease inhibitor tablet (Thermo Scientific)). 500µl of glass beads (Sigma) were added and cells were lysed with the FastPrep-24 (MP Biomedicals) (2x 20 seconds, 4°C). The bottoms of the tubes were pierced with a needle and balanced on top of an eppendorph placed at the bottom of a falcon tube for collection of the sample and centrifuged (3000rcf, 1 minute, 4°C). 275µl of lysis buffer was added to the beads and the centrifugation repeated. The pellet and supernatant was removed from the eppendorph and clarified (25000g, 4°C). This step was repeated on the supernatant and 50µl of the final clarified sample was boiled in 1x Laemmli (95°C, 5 minutes).

5.3.3 Polyacrylamide-gel electrophoresis (SDS-PAGE)

Samples were loaded onto homemade SDS polyacrylamide separating gels (8-10%) with a 4% stacking gel. The boiled samples were run on the gel in SDS running buffer (25mM Tris, 192mM glycine, 0.1% SDS) for 160V for 1 hour, or until the bromophenol blue dye had reached the bottom (whichever came first).

5.3.4 Western blot

Once proteins were separated by SDS-PAGE as previously described, the proteins were transferred onto a PVDF membrane (Millipore) by wet transfer (200V, 2 hours, at 4°C) in transfer buffer (25mM Tris, 1mM EDTA, 20% EtOH, 192 mM glycine). The membrane was then removed from the transfer apparatus and blocked with milk in 5% skim-milk (Sigma) in 1x PBST (1x phosphate-buffered saline, 0.1% tween) for 30 minutes on a shaker. The membrane was incubated with primary antibody (Table 5.4) overnight in 1xPBST at 4°C. The membrane was washed with PBST (3x 10 minutes, shaker) and treated with a horseradish peroxide conjugated secondary antibody (Table 5.4) for 1 hour at room temperature. The membrane was washed again as described previously, and protein was visualised using the clarity ECL substrate (Bio-Rad) and exposed using the Odyssey Fc imaging system (Li-Cor). If the primary antibody was conjugated (-HRP in Table 5.4), the protein was visualised and exposed immediately after washing with PBST after overnight incubation. For control of loading, acidic (pH 2.2) stripping buffer was prepared (200mM glycine, 3mM SDS, 1% tween) and used to strip membranes (2x10 minutes), followed by washing with PBS (2x10 minutes) and TBST (2x5 minutes). Membranes were probed with Tubulin antibody (following the previous instructions for non-conjugated antibodies) or ponceau (0.1% ponceau w/v S, 5% v/v acetic acid).

5.3.5 Northern blot

Preparation of samples: *S. pombe* strains were cultured as previously described. After a 4 hour shift, RNA extraction was carried out as previously described. 8 μ g of RNA was precipitated overnight at -20°C (90 μ l dH₂O, 10 μ l 3M NaAc, 1 μ l glycogen, 250 μ l 100% EtOH). After precipitation, samples were micro centrifuged for 30 minutes (25000g, 4°C) and washed by micro centrifuging for 5 minutes in 70% EtOH (25000g, 4°C). EtOH was removed and samples were allowed to air dry (room-temperature, 5 minutes). The pellet was dissolved in 10 μ l formamide and 1 μ l dH₂O and transferred to ice. 2 μ l of 10X MOPS (200mM MOPS pH 7.0 (3-(N-morpholino)propanesulfonic acid), 80mM NaAc, 30mM EDTA), 4 μ l of formaldehyde, and 1 μ l of dH₂O were added to samples and RNA solutions were incubated at 65°C for 20 minutes. 2 μ l of formaldehyde gel-loading buffer (50% v/v glycerol, 10mM EDTA pH 8.0, bromophenol blue) was added to samples.

Electrophoresis: A 1.2% formaldehyde gel was made by suspending 3.6g of agarose in 216 ml of nuclease-free water and heating in a microwave oven. The solution was allowed to cool to approximately 60°C and then 30ml of 10x MOPS and 54 ml of 37% formaldehyde (2.2 M) was added. The gel was poured into the gel-bed and the sample-comb was inserted. After polymerising, the gel was transferred to the electrophoresis apparatus filled with 1xMOPS. The gel was pre-ran for 5 minutes and then RNA samples were loaded into the wells of the gel and run at 120V for 3 hours.

Transfer: The gel was removed from the container and submerged in NaOH (10mM, 20 minutes, with shaking), removed and replaced with 20X SSC (3M NaCl, 300mM sodium citrate) for 5 minutes, followed by 10X SSC for 40 minutes. RNA was transferred onto a nylon-transfer membrane (Amersham) by capillary transfer (in 10X SSC) and left for 20-24 hours. RNA was immobilised by UV cross-linking (Stratagene UV cross-link, 1200 units) and washed with 3% HAc (10 minutes with shaking). rRNA bands were visualised by staining with methylene blue (0.04% methylene blue, 0.5M NaAc pH 5.2) and imaged with the GelDoc for use as the loading control. Membranes were destained with dH₂O.

Hybridisation and probing: Membranes were immersed in hybridisation solution (20mM Tris-HCl pH 8.0, 0.15mM BSA, 1mM EDTA, 7% SDS, 100mM sodium phosphate buffer pH 8.0), with 200 μ l ssDNA, 10 μ l hybridisation buffer, and incubated at 42°C overnight. The probe was created by PCR and cut out of the gel using a Zymoclean Gel DNA Recovery Kit (Zymo Research). The probe was treated with the Prime-It II Random Primer Labelling Kit by following the manufacturer's instructions: 50ng of the probe was combined with 10 μ l of random hexamer primers and incubated at 95°C for 5 minutes, followed by addition of 10 μ l dATP buffer and 1 μ l Klenow enzyme. In the hot room, 3 μ l of α -P32-dATP was added and incubated at 37°C for 20 minutes. After incubation, the sample was passed through a ProbeQuant G-50 micro column (GE Life Sciences) through centrifuging at 750rcf for 2 minutes, followed by 5 minutes incubation at 95°C. The radioactive sample was added to the membranes with fresh hybridisation buffer and ssDNA and incubated at 42°C overnight. The following day, membranes were washed (2X SSC, 20 minutes) and signal checked with a Geiger counter after each wash. Once the signal was localised to one area of the membrane, the membranes were placed in a cassette along with 2x Amersham Hyperfilm HP films and stored at -80°C until visualised. Visualisation was carried out with the SRX-101A system (Konica Minolta).

5.4 Protein purification

5.4.1 Cell preparation

Cells were grown in YES overnight at 30°C to an OD₆₀₀ = 0.8. Cells were spun down at 4000rpm for 5 minutes at 4°C, followed by spinning down at 4000rpm for 30 minutes. Pellets were flash frozen in liquid nitrogen, ground with a cryogenic grinder (Horiba, 6870) to form a fine powder and stored at -80°C until use.

5.4.2 Flag purification

Pellets were resuspended in lysis buffer (50mM Tris pH 7.5, 150mM NaCl, 10% glycerol, 0.5% triton X-100, 0.5mM DTT, 0.5mM MgCl_2), with the addition of protease inhibitors (1mM PMSF, 10 $\mu\text{g}/\text{ml}$ Aprotinin, 1 $\mu\text{g}/\text{ml}$ Leupeptin, 1 $\mu\text{g}/\text{ml}$ Pepstatin A, 1mM Benzamidine). Resuspended pellet was spun down at 16000g (20 minutes, 4°C) and incubated for 2 hours at 4°C with 150 $\mu\text{l}/1\text{L}$ Anti-Flag M2 affinity gel (Sigma, A2220). After incubation, the resin was washed 4 times for 2 minutes with wash buffer (50mM Tris pH 7.5, 200mM NaCl, 1M Urea, 10% glycerol, 0.5% triton X-100, 0.5mM DTT, 0.5mM MgCl_2). The resin was then washed 4 times for 2 minutes with the same buffer without triton. To elute the protein, samples were incubated twice with 450 μl of 3xFlag peptide (Sigma, F4799) solution for 15 minutes (5mg/ml in Elution buffer: 25mM Tris, 150mM NaCl, 10% glycerol, 0.5mM DTT, 0.5mM MgCl_2 , 0.5mM $\text{Mg}(\text{OAc})_2$). Eluates were passed through BioRad columns to separate the protein from the beads.

5.4.3 Strep purification

The cell powder was prepared from the cell pellet as previously described. The cell powder was dissolved in 5ml/g buffer A (50mM HEPES pH 8.0, 150mM NaCl, 1mM MgCl_2 , 10% glycerol) with the addition of 1mM PMSF, 50U/ μl Benzonase (Sino Biological) and one Pierce protease inhibitor tablet/50ml buffer (Thermoscientific). A magnetic stirrer was used to dissolve the pellet (at 4°C) and make a homogenous solution. The resuspended pellet was spun down at 40000g (1 hour, 4°C). The lysate was filtered (0.22 μm) and pumped directly to a 5ml Strep-Tactin column. The column was washed with 150ml buffer A and eluted by washing with Buffer B (Buffer A, 2.5mM desthiobiotin). Between purifications, the column was reconstituted by washing with 25ml 0.5M NaOH. 15 μl of each of the individual fractions was separated by SDS-PAGE electrophoresis as previously described and stained by coomassie to identify the relevant fractions for the second step purification. The relevant fractions were combined and concentrated with 10K Amicon Ultra-15 centrifugal filter units (Merk) to a final volume of 200 μl . The eluate was imputed

into size exclusion chromatography (gel-filtration) with a superpose 6 10/300 column (GE life sciences) column and washed with 72ml buffer A (3x column volume). Western blot was carried out on 15 μ l of each of the individual fractions with the Strep-Tactin antibody (Table 5.4) and 15 μ l of each fraction was separated on an SDS-polyacrylamide gel and stained by coomassie. The fractions of interest were combined and concentrated with 10K Amicon Ultra-15 centrifugal filter units. The concentrated sample was separated on a precast NuPAGE gel and stained by coomassie.

5.4.4 Silver staining and coomassie

Samples for silver stain were resuspended in Laemmli buffer (12.5mM Tris/HCl pH 6.8, 1% β -mercaptoethanol, 2% glycerol, 0.4% SDS, 0.0005% bromphenolblue) and heated for 5 minutes at 95°C. Proteins were loaded into 1.0mm NuPAGE Bis-Tris precast 4-12% gels (Invitrogen). Gels were run in 1xMOPS (50mM MOPS, 50mM Tris Base, 0.1% SDS, 1mM EDTA, pH 7.7) at 160V for 1 hour. Gels were stained using SilverQuest silver staining kit (Invitrogen). MS was carried out by the Advanced Proteomics Facility, University of Oxford.

Proteins to be visualised by coomassie were separated on a gel as previously described and gels were stained with Instant Blue coomassie Protein Stain (Expedeon) for 15 minutes to 1 hour with shaking, or until bands appeared (whichever came first). Gels were de-stained with dH₂O overnight with shaking.

5.5 RNA sequencing (RNA-seq)

cDNA libraries: All RNA-seq was carried out in duplicate unless otherwise stated. RNA was extracted as previously described. 15ng of RNA was treated with DNase for 1.5 hours at 37°C (20 μ l 10x buffer, 5 μ l RQI enzyme). After DNAase treatment RNA treated with phenol-chloroform (previously described) and precipitated overnight at -20°C. After precipitation, samples were washed with 70% EtOH, air

dried and resuspended in 25 μ l of dH₂O. RNA was quantified by Nanodrop and analysed by Bioanalyzer. 6 μ g of RNA was used for ribo-depletion. Ribo-depletion was carried out using ribominus transcriptome isolation kit (Thermofisher), using the protocol as described by the manufacture. 50ng of bioanalyzer-confirmed ribo-depleted RNA was used for cDNA libraries. Libraries were creating using NEBNext Ultra II Directional RNA library prep kit (Illumina). The protocol was followed as described by the manufacturer. cDNA libraries were confirmed by bioanalyzer.

5.6 Quantification and bioinformatics

5.6.1 RNA sequencing alignment

Reads were aligned using Galaxy (Afgan et al. 2018) and using separate index files for *S. pombe* and the *S. cerevisiae* spike in. After alignment of stranded, paired-end RNA Seq data, the reads in the bam file were separated according to pairs and into .bam and .bedgraph files using script generated by Sina Wittman in the Vasilieva Lab (see code in materials and methods). Data was incorporated into R Studio and differential expression for all genes was analysed using the ‘DESeq2’ package (Love et al. 2014) and referenced against the *S. pombe* annotation from the Cramer lab (Eser et al. 2016). R libraries were normalised for library size. After a list of differentially expressed genes was produced from DESeq, genes were converted into a .csv file and saved for further analysis. Strand-specific .bam files were imported into genome browser and used to scan for visual changes in gene expression and figures were created with snapshots of genome browser.

5.7 Quantification of microscopy

Live-cell microscopy: ImageJ was used to quantify mean fluorescence signal seen in GFP live-cell microscopy. The DAPI signal was used to take a fixed area around the

nucleus and the DIC images were used as a reference of the outline of the cell to take a fixed area in the cytoplasm. The fixed area was mapped on poly(A) images and used to measure the mean fluorescence intensity on the GFP filter in the nucleus and the cytoplasm. The mean fluorescent intensity was used to create a ratio of the signal in the nucleus to that in the cytoplasm for each strain.

FISH microscopy quantification: ImageJ was used to quantify median fluorescence signal. The DAPI signal was used to take a fixed area around the nucleus and the DIC images were used for reference of the outline of the cell to take a fixed area in the cytoplasm. The fixed area was mapped on poly(A) images and used to measure the median fluorescence intensity on the GFP filter in the nucleus and the cytoplasm. The median fluorescent intensity was used to create a ratio of the signal in the nucleus to that in the cytoplasm for each strain.

5.8 Code

All statistical analysis was carried out by myself and using R Studio.

5.8.1 Code for RNA sequencing analysis

Example showing separation of reads, conversion into .bam and .bedgraph for IGB and DESeq analysis. Example shown for YP71 (wild-type) at 25°C compared to 37°C. The same process was repeated for all samples included in RNA-seq.

#separation of galaxy produced reads according to pairs:

```
samtools view -h YP71-25c_rep2_pombe_UniqMapped.bam  
| awk 'substr($0,1,1) == "@" {print}  
{if($2 == 147 || $2 == 99) print}'  
| samtools view -bS > 71-25c_minus.bam
```

```
samtools view -h YP71-25c_rep2_pombe_UniqMapped.bam
| awk 'substr($0,1,1) == "@" {print}
{if($2 == 163 || $2 == 83) print}'
| samtools view -bS > 71-25c_plus.bam
```

#converting the resulting files into bam format:

```
samtools sort -T /tmp/aln.sorted -o
71-25c_minus_sorted.bam 71-25c_minus.bam
```

```
samtools sort -T /tmp/aln2.sorted -o
71-25c_plus_sorted.bam 71-25c_plus.bam
```

The files are converted into bedgraphs

```
bedtools genomecov -ibam 71-25c_minus_sorted.bam
-bg -scale 0.323048205 > 71-25c_minus.bedgraph
```

```
bedtools genomecov -ibam 71-25c_plus_sorted.bam
-bg -scale 0.323048205 > 71-25c_plus.bedgraph
```

DESeq analysis

```
library('DESeq2')
setwd("/Volumes/TRANSCEND/Studies/RNA-seq/bam/")
f_name <- "./fc_71-67-1416_allTUs.csv"

fc_data <- read.table(f_name, header=TRUE, row.names=1)
fc_data

names(fc_data) <- c("Chr", "Start", "End", "Strand", "Length",
" type", "Gene", "71-25_rep1", "71-37_rep1", "67-25_rep1",
"67-37_rep1", "1416-25_rep1", "1416-37_rep1", "71-25_rep2",
"71-37_rep2", "67-25_rep2", "67-37_rep2", "1416-25_rep2",
```

```

"1416_37_rep2")

countdata <- subset(fc_data, select=c("71_25_rep1", "71_25_rep2",
    "71_37_rep1", "71_37_rep2"))

countdata
countdata <- as.matrix(countdata)
condition <- c("71_25", "71_25", "71_37", "71_37")
coldata <- data.frame(row.names=colnames(countdata), condition)
dds <- DESeqDataSetFromMatrix(countData = countdata, colData = coldata,
    design = ~ condition)
dds
dds <- dds[ rowSums(counts(dds)) > 5, ]
dds
dds$condition <- relevel(dds$condition, ref = "71_25")
dds$condition
sizeFactors(dds) <- c(1,1.154037838,1.260016146,1.154037838)
norm <- counts(dds, normalized=TRUE)
dds <- estimateDispersions(dds)
dds = nbinomWaldTest(dds)
res = results(dds, alpha=0.05)
summary(res)
res

plotMA(res, ylim=c(-8,8))

counts(dds)
norm
write.csv(norm, file="./normalized_counts_71_25_37_allTUs.csv")
write.csv(res, file = "./DE_71_25_37_allTUs.csv")

#adding gene IDs to TU values for analysis

genename <- read.csv('gene_names.csv')

```

```

colnames(genename) <- c("ID", "Type", "Gene")
head(genename)
wt <- read.csv('wt.csv')

colnames(wt) <- c("ID")
wt_by_name <- merge(wt, genename, by='ID')
write.csv(wt_by_name, file="wt_by_name.csv")

```

5.8.2 Code for FISH image analysis

Statistical analysis on median values for ratio of fluorescence from fish images in Section 2.5. Repeated measures ANOVA was carried out to calculate differences between strains of the same temperature (e.g. 25°C wild-type vs 25°C *dis3-54*) and differences between scores of temperatures (e.g. 25°C wild-type vs 37°C wild-type).

```

fish <- read.csv("fishmedian.csv")
str(fish)
fish$temp <- factor(fish$temp,
                    levels = c(25, 37),
                    labels = c("25", "37"))
str(fish)
anova_fish <- aov(score ~ strain + temp, data = fish)
summary(anova_fish)

#strain Pr(>F) = <2e-16 ***
#temp Pr(>F) = 0.0066 **

TukeyHSD(fish)

#p adj
#Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05

```

#doubled:25-dis3:25	0.0000000	***
#doubler:25-dis3:25	0.0000000	***
#rrp6:25-dis3:25	0.0925409	ns
#sbp1:25-dis3:25	0.0065524	**
#wt:25-dis3:25	0.0013462	**
#dis3:37-dis3:25	0.0410761	*
#doubler:25-doubled:25	0.0000000	***
#rrp6:25-doubled:25	0.0027641	**
#sbp1:25-doubled:25	0.0000000	***
#wt:25-doubled:25	0.0000000	***
#doubled:37-doubled:25	1.0000000	ns
#rrp6:25-doubler:25	0.0000000	***
#sbp1:25-doubler:25	0.0000000	***
#wt:25-doubler:25	0.0000000	***
#doubler:37-doubler:25	0.0000000	***
#sbp1:25-rrp6:25	0.0000000	***
#wt:25-rrp6:25	0.0000000	***
#rrp6:37-rrp6:25	0.0000022	***
#wt:25-sbp1:25	0.9999998	ns
#sbp1:37-sbp1:25	0.0000000	***
#wt:37-wt:25	1.0000000	ns
#doubled:37-dis3:37	0.0000000	***
#doubler:37-dis3:37	0.0000000	***
#rrp6:37-dis3:37	0.0000000	***
#sbp1:37-dis3:37	0.0000000	***
#wt:37-dis3:37	0.9998950	ns
#doubler:37-doubled:37	1.0000000	ns
#rrp6:37-doubled:37	0.8826939	ns
#sbp1:37-doubled:37	0.3239875	ns
#wt:37-doubled:37	0.0000000	***
#rrp6:37-doubler:37	0.9638130	ns
#sbp1:37-doubler:37	0.5062126	ns
#wt:37-doubler:37	0.0000000	***
#sbp1:37-rrp6:37	0.9993887	ns

```
#wt:37-rrp6:37          0.0000000 ***
#wt:37-sbp1:37          0.0000000 ***
```

5.8.3 Code for GFP image analysis

Statistical analysis on mean values for ratio of fluorescence from fish images in Section 2.11. One-way ANOVA was carried out to calculate differences between the mean fluorescence values of Sbp1-GFP and 1-GFP in exosome mutants (*dis3-54*, *rrp6Δ*, or *mtl1-1*).

```
gfp <- read.csv('gfp.csv')
shapiro.test(gfp$score)

res.aov <- aov(score ~ strain, data = gfp)
res.aov
summary(res.aov)

#strain Pr(>F) = 2.66e-13 ***

TukeyHSD(res.aov2)

#p adj
#gfp-dis354      0.0000061 ***
#mtl11-dis354    0.0000328 ***
#rrp6d-dis354    0.9414086 ns
#mtl11-gfp       0.0000000 ***
#rrp6d-gfp       0.0000194 ***
#rrp6d-mtl11     0.0000099 ***
```


Bibliography

- Afgan, E., Baker, D., Batut, B., van-den Beek, M., Bouvier, D., Čech, M., Chilton, J., Clements, D., Coraor, N., Grüning, B., Guerler, A., Hillman-Jackson, J., Jalili, V., Rasche, H., Soranzo, N., Goecks, J., Taylor, J., Nekrutenko, A. & Blankenberg, D. (2018), ‘The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update’, *Nucleic Acids Res.* **46**(W1), W537–W544.
- Aguilera, A. & Garcia-Muse, T. (2012), ‘R loops: from transcription byproducts to threats to genome stability’, *Mol. Cell.* **46**(2), 115–124.
- Allmang, C., Kufel, J., Chanfreau, G., Mitchell, P., Petfalski, E. & Tollervey, D. (1999), ‘Functions of the exosome in rRNA, snoRNA and snRNA synthesis’, *EMBO J* **18**(19), 5399–5410.
- Assenholt, J., Mouaikel, J., Andersen, K., Brodersen, D., Libri, D. & Jensen, T. (2008), ‘Exonucleolysis is required for nuclear mRNA quality control in yeast THO mutants’, *RNA* **14**(11), 2305–2313.
- Becker, D., Hirsch, A., Bender, L., Lingner, T., Salinas, G. & Krebber, H. (2019), ‘Nuclear pre-snRNA export is an essential quality assurance mechanism for functional spliceosomes.’, *Cell Rep.* **27**(11), 3214.
- Bentley, D. (2014), ‘Coupling mRNA processing with coupling mRNA processing with transcription in time and space’, *Nat. Rev. Genet.* **15**, 163–175.
- Biancheri, R., Cassandrini, D., Pinto, F., Trovato, R., Rocco, M., mirabelli Badenier, M., Pedemonte, M., Panicucci, C., Trucks, H., Sander, T., Zara, F., Rossi, A., Striano, P., Minetti, C. & Santorelli, F. (2013), ‘EXOSC3 mutations in isolated cerebellar hypoplasia and spinal anterior horn involvement’, *J. Neurol.* **260**(7), 1866–1870.
- Biro, A., Kilchert, C., Kus, K., Priest, E., Alwash, A., Heo, D., Castello, A., Mohammed, S. & Vasiljeva, L. (unpublished manuscript from the Vasilieva lab, 2020),

- ‘Comprehensive analysis of RNA-bound proteomes from fission yeast mutants of the nuclear exosome’, *BioRxiv*.
- Bischoff, F., Klebe, C., Kretschmer, J., Wittinghofer, A. & Ponstingl, H. (1994), ‘RanGAP1 induces GTPase activity of nuclear Ras-related Ran’, *Proc. Natl. Acad. Sci. U. S. A.* **91**, 2587–2591.
- Bischoff, F., Krebber, H., Kempf, T., Hermes, I. & Ponstingl, H. (1995), ‘Human RanGTPase-activating protein RanGAP1 is a homologue of yeast Ran1p involved in mRNA processing and transport’, *Proc. Natl. Acad. Sci.* **92**, 1749–1753.
- Bischoff, F. & Ponstingl, H. (1991*a*), ‘Catalysis of guanine nucleotide exchange on ran by the mitotic regulator RCC1’, *Nature* **354**(6348), 80–2.
- Bischoff, F. & Ponstingl, H. (1991*b*), ‘Mitotic regulator protein RCC1 is complexed with a nuclear Ras-related polypeptide’, *Proc. Natl. Acad. Sci. U. S. A.* **88**(23), 10830–10834.
- Bitton, D., Atkinson, S., Rallis, C., Smith, G., Ellis, D., Chen, Y., Malecki, M., Codlin, S., Lemay, J., Cotobal, C., Bachand, F., Marguerat, S., Mata, J. & Bahler, J. (2015), ‘Widespread exon skipping triggers degradation by nuclear RNA surveillance in fission yeast.’, *Genome Res.* **25**(6), 884–896.
- Boczonadi, V., Müller, J., Pyle, A., Munkley, J., Dor, T., Quartararo, J., Ferrero, I., Karcagi, V., Giunta, M. & Polvikoski, T. (2014), ‘EXOSC8 mutations alter mRNA metabolism and cause hypomyelination with spinal muscular atrophy and cerebellar hypoplasia’, *Nat. Commun.* **4287**.
- Bousquet-Antonelli, C., Presutti, C. & Tollervey, D. (2000), ‘Identification of a regulated pathway for nuclear pre-mRNA turnover’, *Cell* **102**(765-775).
- Bresson, S. & Conrad, N. (2013), ‘The human nuclear poly(A)-binding protein promotes RNA hyperadenylation and decay’, *PloS Genet.* **9**.
- Bronner, C., Salvi, L., Zocco, M., Ugolini, I. & Halic, M. (2017), ‘Accumulation of RNA on chromatin disrupts heterochromatic silencing’, *Genome Res.* **27**(7), 1174–1183.
- Bühler, M., Haas, W., Gygi, S. & Moazed, D. (2007), ‘RNAi-dependent and independent RNA turnover mechanisms contribute to heterochromatic gene silencing’, *Cell* **129**, 707–721.

- Bühler, M., Spies, N., Bartel, D. & Moazed, D. (2008), ‘TRAMP-mediated RNA surveillance prevents spurious entry of RNAs into the *Schizosaccharomyces pombe* siRNA pathway’, *Nat. Struct. Mol. Biol.* **15**, 1015–1023.
- Burns, D., Donkervoort, S., Müller, J., Knierim, E., Bharucha-Goebel, D., Fageih, E., Bell, S., AlFaifi, A., Monies, D., Millan, F., Retterer, K., Dyack, S., MacKay, S., Morales-Gonzalez, S., Giunta, M., Munro, B., Hudson, G., Scavina, M., Baker, L., Massini, T., Lek, M., Hu, Y., Ezzo, D., AlKuraya, F., Kang, P., Griffin, H., Foley, R., Schuelke, M., Horvath, R. & Bönnemann, C. (2018), ‘Variants in EXOSC9 disrupt the RNA exosome and result in cerebellar atrophy with spinal motor neuronopathy’, *Am. J. Hum. Genet.* **102**(5), 858–873.
- Cáceres-Gutiérrez, R. & Herrera, L. (2017), ‘Centromeric non-coding transcription: opening the black box of chromosomal instability?’, *Curr. Genomics* **18**(3), 227–235.
- Cai, X., Hagedorn, C. & Cullen, B. (2004), ‘Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs’, *RNA* **10**(12), 1957–1966.
- Carneiro, T., Carvalho, C., Braga, J., Rino, J., Milligan, L. & Tollervey, D. (2007), ‘Depletion of the yeast nuclear exosome subunit Rrp6 results in accumulation of polyadenylated RNAs in a discrete domain within the nucleolus’, *Mol. Cell. Biol.* **27**(11), 4157–4165.
- Carobbio, S., Realini, C., Norbury, C., Toda, T., Cavalli, F. & Spataro, V. (2001), ‘Sequence of Crm1/exportin 1 mutant alleles reveals critical sites associated with multidrug resistance’, *Curr. Genet.* **39**(2-9).
- Castello, A., Horos, R., Strein, C., Fischer, B., Eichelbaum, K., Steinmetz, L., Krijgsveld, J. & Hentze, M. (2013), ‘System-wide identification of RNA-binding proteins by interactome capture’, *Nature Protocols* **8**, 491–500.
- Cavazza, T. & Vernos, I. (2016), ‘The RanGTP pathway: from nucleo-cytoplasmic transport to spindle assembly and beyond’, *Front. Cell. Dev. Biol.* **3**(82).
- Choi, E., Strålfors, A., Castillo, A., Durand-Dubief, M., Ekwall, K. & Allshire, R. (2011), ‘Identification of noncoding transcripts from within CENP-A chromatin at fission yeast centromeres’, *J. Biol. Chem.* **286**(26), 23600–23607.
- Coy, S., Volanakis, A., Shah, S. & Vasiljeva, L. (2013), ‘The Sm complex is required for the processing of non-coding RNAs by the exosome’, *PloS One* **8**(6).

- Davidson, L., Francis, L., Cordiner, R., Eaton, J., Estell, C., Macias, S., Caceres, J. & West, S. (2019), ‘Rapid depletion of DIS3, EXOSC10, or XRN2 reveals the immediate impact of exoribonucleolysis on nuclear RNA metabolism and transcriptional control’, *Cell Rep.* **26**(10), 2779–2791.
- Davis, C. & Ares, M. (2006), ‘Accumulation of unstable promoter-associated transcripts upon loss of the nuclear exosome subunit Rrp6p in *Saccharomyces cerevisiae*’, *Proc. Natl. Acad. Sci.* **103**, 3262–3267.
- Ding, D., Okamasa, K., Katou, Y., Oya, E., Nakayama, J., Chikashige, Y., Shirahige, K., Haraguchi, T. & Hiroaka, Y. (2019), ‘Chromosome-associated RNA-protein complexes promote pairing of homologous chromosomes during meiosis in *Schizosaccharomyces pombe*’, *Nat Commun.* **10**.
- Ding, D., Okamasa, K., Yamane, M., Tsutsumi, C., Haraguchi, T., Yamamoto, M. & Hiraoka, Y. (2012), ‘Meiosis-specific noncoding RNA mediates robust pairing of homologous chromosomes in meiosis’, *Science* **336**(6082), 732–736.
- Dirks, R., Molenaar, C. & Tanke, H. (2003), ‘Visualizing RNA molecules inside the nucleus of living cells’, *Methods* **29**(1), 51–57.
- Dziembowski, A., Lorentzen, E., Conti, E. & Séraphin, B. (2007), ‘A single subunit, Dis3, is essentially responsible for yeast exosome core activity’, *Nat Struct Mol Biol* **14**, 15–22.
- Egan, E., Braun, C., Gygi, S. & Moazed, D. (2014), ‘Post-transcriptional regulation of meiotic genes by a nuclear RNA silencing complex’, *RNA* **20**(6), 867–881.
- Eser, P., Wachutka, L., Maier, K., Demel, C., Boroni, M., Lyer, S., Cramer, P. & Gagneur, J. (2016), ‘Determinants of RNA metabolism in the *Schizosaccharomyces pombe* genome’, *Mol. Syst. Biol.* **12**(2), 857.
- Fan, J., Kuai, B., Wu, G., Wu, X., Chi, B., Wang, L., Wang, K., Shi, Z., Zhang, H., Chen, S., He, Z., Wang, S., Zhou, Z., Li, G. & Cheng, H. (2017), ‘Exosome cofactor hMTR4 competes with export adaptor ALYREF to ensure balanced nuclear RNA pools for degradation and export’, *EMBO J* **36**(19), 2870–2886.
- Fischer, U. & Luhrmann, R. (1990), ‘An essential signaling role for the m3G cap in the transport of U1 snRNP to the nucleus’, *Science* **249**, 785–790.

- Fleig, U., Salus, S., Karig, I. & Sazer, S. (2000), ‘The fission yeast Ran GTPase is required for microtubule integrity’, *J. Cell. Biol.* **151**(5), 1101–1112.
- Forrester, W., Stutz, F., Rosbash, M. & Wickens, M. (1992), ‘Defects in mRNA 3’-end formation, transcription initiation, and mRNA transport associated with the yeast mutation *prp20*: possible coupling of mRNA processing and chromatin structure’, *Genes Dev.* **6**(10), 1914–1926.
- Fox, M., Gao, H., Smith-Kinnaman, W., Liu, Y. & Mosley, A. (2015), ‘The exosome component Rrp6 is required for RNA polymerase II termination at specific targets of the Nrd1-Nab3 pathway’, *PloS Genet.* **11**.
- Garland, W., Comet, I., Wu, M., Radzischeuskaya, A., Rib, L., Vitting-seerup, K., Lloret-Llinares, M., Sandelin, A., Helin, K. & Jensen, T. (2019), ‘A functional link between nuclear RNA decay and transcriptional control mediated by the polycomb repressive complex 2’, *Cell Rep.* **29**(7), 1800–1811.e6.
- Gopal, G., Yoon, J., Kozak, L., Thakurta, A. & Dhar, R. (2005), ‘Homolog of BRCA2-interacting Dss1p and Uap56p link Mlo3p and Rae1p for mRNA export in fission yeast’, *EMBO J* **24**(14), 2512–2523.
- Hardwick, S. & Luisi, B. (2013), ‘Rarely at rest RNA helicases and their busy contributions to RNA degradation, regulation and quality control’, *RNA Biol.* **10**(1), 56–70.
- Harigaya, Y., Tanaka, H., Yamanaka, S., Tanaka, K., Watanabe, Y. & Tsutsumi, C. (2006), ‘Selective elimination of messenger RNA prevents an incidence of untimely meiosis’, *Nature* **442**(7098), 45–50.
- He, X., Hayashi, N., Walcott, N., Azuma, Y., Patterson, T. & Bischoff, F. (1998), ‘The identification of cDNAs that affect the mitosis-to-interphase transition in *Schizosaccharomyces pombe*, including Sbp1, which encodes a Spilp-GTP-binding protein’, *Genetics* **148**(2), 645–656.
- Hilleren, P., McCarthy, T., Rosbash, M., Parker, R. & Jensen, T. (2001), ‘Quality control of mRNA 3’-end processing is linked to the nuclear exosome’, *Nature* **04**(413), 6855.
- Hilleren, P. & Parker, R. (2001), ‘Defects in the mRNA export factors Rat7p, Gle1p, Mex67p and Rat8p cause hyperadenylation during 3’-end formation of nascent transcripts’, *RNA* **7**, 753–764.

- Hiriart, E., Vavasseur, A., Touat-Todeschini, L., Yamashita, A., Gilquin, B. & Lambert, E. (2012), ‘Mmi1 RNA surveillance machinery directs RNAi complex RITS to specific meiotic genes in fission yeast’, *EMBO J* **31**(10), 2296–2308.
- Hopper, A., Banks, F. & Evangelidis, V. (1978), ‘A yeast mutant which accumulates precursor tRNAs’, *Cell* **14**(2), 211–219.
- Hu, B., Petela, N., Kurze, A., Chan, K., Chapard, C. & Nasmyth, K. (2015), ‘Biological chromodynamics: a general method for measuring protein occupancy across the genome by calibrating ChIP-seq’, *Nucleic Acids Res.* **43**(20).
- Hulsen, T., D-Vleig, J. & Alkema, W. (2008), ‘BioVenn - a web application for the comparison and visualisation of biological lists using area-proportional Venn diagrams’, *BMC Genomics* **9**(1), 488.
- Jensen, T., Patricio, K., McCarthy, T. & Rosbash, M. (2001), ‘A block to mRNA nuclear export in *S. cerevisiae* leads to hyperadenylation of transcripts that accumulate at the site of transcription’, *Mol. Cell.* **7**(4), 887–898.
- Jia, H., Wang, X., Liu, F., Guenther, U., Srinivasan, S., Anderson, J. & Jankowski, E. (2011), ‘The RNA helicase Mtr4p modulates polyadenylation in the TRAMP complex’, *Cell* **145**(6), 890–901.
- Jimeno, S., Rondon, A., Luna, R. & Arguilera, A. (2002), ‘The yeast THO complex and mRNA export factors link RNA metabolism with transcription and genome instability’, *EMBO J* **21**(13), 3536–3535.
- Kadowaki, T., Goldfarb, D., Spitz, L., Tartakoff, A. & Ohno, M. (1993), ‘Regulation of RNA processing and transport by a nuclear guanine nucleotide release protein and members of the Ras superfamily’, *EMBO J* **12**(7), 2929–2937.
- Kehlenback, R., Dickmanns, A., Kehlenback, A., Guan, T. & Gerace, L. (1999), ‘A role for RanBP1 in the release of CRM1 from the nuclear pore complex in a terminal step of nuclear export’, *J. Cell. Biol.* **145**(4), 645–657.
- Keller, C., Woolcock, K., Hess, D. & Bühler, M. (2010), ‘Proteomic and functional analysis of the noncanonical poly(A) polymerase Cid14’, *RNA* **16**(6), 1124–1129.
- Kelley, L., Mezulis, S., Yates, C., Wass, M. & Steinberg, M. (2015), ‘The Phyre2 web portal for protein modeling, prediction and analysis’, *Nature Protocols* **10**, 845–858.

- Kilchert, C., Kecman, T., Priest, E., Hester, S., Kus, K., Castello, A., Mohammed, S. & Vasiljeva, L. (BioRxiv, 2019, accepted to Genome Research 2020)), ‘System-wide analyses of the fission yeast poly(A)+ RNA interactome reveal insights into organisation and function of RNA-protein complexes’, *BioRxiv* .
- Kilchert, C., Wittmann, S., Passoni, M., Shah, S., Granneman, S. & Vasiljeva, L. (2015), ‘Regulation of mRNA levels by decay-promoting introns that recruit the exosome specificity factor Mmi1’, *Cell Rep.* **13**, 2504–2515.
- Kilchert, C., Wittmann, S. & Vasiljeva, L. (2016), ‘The regulation and functions of the nuclear RNA exosome complex’, *Nat Rev Mol Cell Biol* **17**, 227–239.
- Kim, D., Hayles, J., Kim, D., Wood, V., Park, H., Won, M., Yoo, H., Duhig, T., Nam, M., Palmer, G., Han, S., Jeffery, L., Baek, S., Lee, H., Shim, Y., Lee, M., Kim, L., Heo, K., Noh, E., Lee, A., Jang, Y., Chung, K., Choi, S., Park, J., Park, Y., Kim, H., Park, S., Park, H., Kang, E., Kim, H., Kang, H., Park, H., Kim, K., Song, K., Song, K., Nurse, P. & Hoe, K. (2010), ‘Analysis of a genome-wide set of gene deletions in the fission yeast *Schizosaccharomyces pombe*’, *Nat. Biotechnol.* **28**(6), 616–623.
- Kinoshita, N., Goebel, M. & Yanagida, M. (1991), ‘The fission yeast *dis3+* gene encodes a 110-kDa essential protein implicated in mitotic control’, *Mol. Cell. Biol.* **11**, 5839–5847.
- Kiss, D. & Andrusis, E. (2010), ‘Genome-wide analysis reveals distinct substrate specificities of Rrp6, Dis3, and core exosome subunits’, *RNA* **16**(4), 781–791.
- Kohler, A. & Hurt, E. (2007), ‘Exporting RNA from the nucleus to the cytoplasm’, *Nat Rev Mol Cell Biol* **8**(10), 761–773.
- Kowalinski, E., Kögel, A., Ebert, J., Reichelt, P., Stegmann, E., Habermann, B. & Conti, E. (2016), ‘Structure of a cytoplasmic 11-subunit RNA exosome complex’, *Mol. Cell.* **63**(1), 125–134.
- Krchnaková, Z., Thakur, P., Krausová, M., Bieberstein, N., Haberman, N., Müller-McNicoll, M. & Stanek, D. (2019), ‘Splicing of long non-coding RNAs primarily depends on polypyrimidine tract and 5’ splice-site sequences due to weak interactions with SR proteins’, *Nucleic Acids Res.* **47**(2), 911–928.
- Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E., Wolff, B., Yoshida, M. & Horinouchi, S. (1999), ‘Leptomycin B inactivates CRM1/exportin 1 by covalent

- modification at a cysteine residue in the central conserved region’, *Proc. Natl. Acad. Sci. U. S. A.* **96**(16), 9112–9117.
- Kunzler, M., Gerstberger, T., Stutz, F., Bischoff, F. & Hurt, E. (2000), ‘Yeast Ran-binding protein 1 (Yrb1) shuttles between the nucleus and cytoplasm and is exported from the nucleus via a CRM1 (XPO1)-dependent pathway’, *Mol. Cell. Biol.* **20**(12), 4295–4308.
- LaCava, J., Houseley, J., Saveanu, C., Petfalski, E., Thompson, E. & Jacquier, A. (2005), ‘RNA degradation by the exosome is promoted by a nuclear polyadenylation complex’, *Cell* **121**(5), 713–724.
- Lee, N., Chalamcharla, V., Reyes-Turcu, F., Mehta, S., Zofall, M. & Balachandran, V. (2013), ‘Mtr4-like protein coordinates nuclear RNA processing for heterochromatin assembly and for telomere maintenance’, *Cell* **155**(5), 1061–1074.
- Lemay, J., D’Amours, A., Lemieux, C., Lackner, D., St-Sauveur, V. & Bahler, J. (2010), ‘The nuclear poly(A)-binding protein interacts with the exosome to promote synthesis of noncoding small nucleolar RNAs’, *Mol. Cell.* **37**(1), 34–45.
- Lemay, J., Larochelle, M., Marguerat, S., Atkinson, S., Bahler, J. & Bachand, F. (2014), ‘The RNA exosome promotes transcription termination of backtracked RNA polymerase II’, *Nat. Struct. Mol. Biol.* **21**, 919–926.
- Lemieux, C., Marguerat, S., Lafontaine, J., Barbezier, N., Bahler, J. & Bachand, F. (2011), ‘A pre-mRNA degradation pathway that selectively targets intron-containing genes requires the nuclear poly(A)-binding protein’, *Mol. Cell.* **44**(1), 108–119.
- Lingaraju, M., Johnsen, D., Schlundt, A., Langer, L., Basquin, J., Sattler, M., Jensen, T., Falk, S. & Conti, E. (2019), ‘The MTR4 helicase recruits nuclear adaptors of the human RNA exosome using distinct arch-interacting motifs’, *Nat. Commun.* **10**(1), 3393.
- Liu, Q., Greimann, J. & Lima, C. (2006), ‘Reconstitution, activities, and structure of the eukaryotic RNA exosome’, *Cell* **127**, 1223–1237.
- Lloret-Llinares, M., Karadoulama, E., Chen, Y., Wojenski, L., Villafano, G., Bornholdt, J., Andersson, R., Core, L., Sandelin, A. & Jensen, T. (2018), ‘The RNA exosome contributes to gene expression regulation during stem cell differentiation’, *Nucleic Acids Res.* **46**(21), 11502–11513.

- Lock, A., Rutherford, K., Harris, M., Hayles, J., Oliver, S., Bahler, J. & Wood, V. (2018), ‘PomBase 2018: user-driven reimplementations of the fission yeast database provides rapid and intuitive access to diverse, interconnected information’, *Nucleic Acids Res.* .
- Love, M., Huber, W. & Anders, S. (2014), ‘Moderated estimation of fold change and dispersion for RNA-seq data with ‘DESeq2’’, *Genome Biol.* **15**(550).
- Lubas, M., Andersen, P., Dziembowski, A., Kudla, G. & Jensen, T. (2015), ‘The human nuclear exosome targeting complex is loaded onto newly synthesized RNA to direct early ribonucleolysis’, *Cell Rep.* **10**(2), 178–192.
- Lubas, M., Christensen, M., Kristiansen, M., Domanski, M., Falkenby, L. & Lykke-Andersen, S. (2011), ‘Interaction profiling identifies the human nuclear exosome targeting complex’, *Mol. Cell.* **1943**(4), 624–637.
- Makino, D. L., Baumgärtner, M. & Conti, E. (2013), ‘Crystal structure of an RNA-bound 11-subunit eukaryotic exosome complex’, *Nature* **495**, 70–75.
- Matsumoto, T. & Beach, D. (1991), ‘Sp1 GTPase interacts with RCC1 to maintain interdependency of cell cycle events’, *Int. Symp.* **22**, 145–152.
- McPheeters, D., Cremona, N., Sunder, S., Chen, H., Averbeck, N., Leatherwood, J. & Wise, J. (2009), ‘A complex gene regulatory mechanism that operates at the nexus of multiple RNA processing decisions’, *Nat Struct Mol Biol* **16**(3), 255–264.
- Melchior, F., Weber, K. & Gerke, V. (1993), ‘A functional homologue of the RNA1 gene product in *Schizosaccharomyces pombe*: purification, biochemical characterization, and identification of a leucine-rich repeat motif’, *Mol. Cell. Biol.* **4**(6), 569–581.
- Meola, N., Domanski, M., Karadoulama, E., Andersen, J., Sandelin, A. & Jensen, T. (2016), ‘Identification of a nuclear exosome decay pathway for processed transcripts’, *Mol. Cell.* **64**(3), 520–533.
- Mercier, A. & Labbe, S. (2009), ‘Both Php4 function and subcellular localization are regulated by iron via a multistep mechanism involving the glutaredoxin Grx4 and the exportin Crm1’, *J. Biol. Chem.* **284**(30), 20249–20262.
- Mikolaskova, B., Jurcik, M., Cipakova, I., Kretova, M., Chovanec, M. & Cipak, L. (2018), ‘Maintenance of genome stability: the unifying role of interconnections between the DNA damage response and RNA-processing pathways’, *Curr. Genet.* **64**(5), 971–983.

- Mistry, D., Chen, Y. & Sen, G. (2012), ‘Progenitor function in self-renewing human epidermis is maintained by the exosome’, *Cell Stem Cell* **11**(1), 127–135.
- Mitchell, P., Petfalski, E., Shevchenko, A., Mann, M. & Tollervey, D. (1997), ‘The exosome: a conserved eukaryotic RNA processing complex containing multiple 3’-5’ exoribonucleases’, *Cell* **91**(4), 457–466.
- Mouaikel, J., Verheggen, C., Bertrand, E., Tazi, J. & Bordonné, R. (2002), ‘Hypermethylation of the cap structure of both yeast snRNAs and snoRNAs requires a conserved methyltransferase that is localized to the nucleolus’, *Mol. Cell.* **9**, 891–901.
- Murakami, H., Goto, D., Toda, T., Chen, E., Grewal, S., Martienssen, R. & Yanagida, M. (2007), ‘Ribonuclease activity of Dis3 is required for mitotic progression and provides a possible link between heterochromatin and kinetochore function’, *PloS One* **2**(3).
- Neil, H., Malabat, C., d’Aubenton Carafa, Y., Xu, Z., Steinmetz, L. & Jacquier, A. (2009), ‘Widespread bidirectional promoters are the major source of cryptic transcripts in yeast’, *Nature* **457**(7232), 1038–42.
- Neville, M. & Rosbash, M. (1999), ‘The NES-Crm1p export pathway is not a major mRNA export route in *Saccharomyces cerevisiae*’, *EMBO J* **18**(13), 3746–3756.
- Noguchi, E., Hayashi, N., Azuma, Y., Seki, T., Nakamura, M. & Nakashima, N. (1996), ‘Dis3, implicated in mitotic control, binds directly to Ran and enhances the GEF activity of RCC1’, *EMBO J* **15**(20), 5595–5605.
- Novoa, I., Rush, M. & D’Eustachio, P. (1999), ‘Isolated mammalian and *Schizosaccharomyces pombe* Ran-binding domains rescue *S. pombe* Sbp1 (RanBP1) genomic mutants’, *Mol. Cell. Biol.* **10**(7), 2175–2190.
- Ohno, M., Segref, A., Bachi, A., Wilm, M. & Mattaj, I. (2000), ‘PHAX, a mediator of U snRNA nuclear export whose activity is regulated by phosphorylation’, *Cell* **101**(2), 187–198.
- Paul, B. & Montpetit, B. (2016), ‘Altered RNA processing and export lead to retention of mRNAs near transcription sites and nuclear pore complexes or within the nucleolus’, *Mol. Cell. Biol.* **27**(17), 2742–2756.

- Perreault, A., Lemieux, C. & Bachand, F. (2007), ‘Regulation of the nuclear poly(A)-binding protein by arginine methylation in fission yeast’, *J. Biol. Chem.* **282**(10), 7552–7562.
- Quinn, J. & Chang, H. (2017), ‘Unique features of long non-coding RNA biogenesis and function’, *Nat. Rev.* **17**, 47–62.
- Reyes-Turcu, F., Zhang, K., Zofall, M., Chen, E. & Grewal, S. (2011), ‘Defects in RNA quality control factors reveal RNAi-independent nucleation of heterochromatin’, *Nat Struct Mol Biol* **18**(10), 1132–1138.
- Rougemaille, M., Gudipati, R., Olesen, J., Thomsen, R., Seraphin, B., Libri, D. & Jensen, T. (2007), ‘Dissecting mechanisms of nuclear mRNA surveillance in THO/subs2 complex mutants’, *EMBO J* **26**, 2317–2326.
- Roundtree, I., Evans, M., Tao, P. & Chuan, H. (2015), ‘Dynamic RNA modifications in gene expression regulation’, *Cell* **169**(7), 1187–1200.
- Rudnik-Schöneborn, S., Senderek, J., Jen, J., Houge, G., Seeman, P., Puchmajerová, A., Graul-Neumann, L., Seidel, U., Korinthenberg, R., Kirschner, J., Seeger, J., Ryan, M., Muntoni, F., Steinlin, M., Sztriha, L., Hüber, C., Brockmann, K., Van Maldergem, L., Schiff, M., Holzinger, A., Barth, P., Reardon, W., Yourshaw, M., Nelson, S., Eggermann, T. & Zerres, K. (2013), ‘Pontocerebellar hypoplasia type 1: clinical spectrum and relevance of EXOSC3 mutations’, *Neurology* **80**(5), 438–446.
- Sabatino, S. & Forsburg, S. (2010), ‘Molecular genetics of *Schizosaccharomyces pombe*’, *Method. Enzymol.* **470**, 761–765.
- Santos-Rosa, H., Moreno, H., Simos, G., Segref, A., Fahrenkrog, B. & Pante, N. (1998), ‘Nuclear mRNA export requires complex formation between Mex67p and Mtr2p at the nuclear pores’, *Mol. Cell. Biol.* **18**(11), 6826–6838.
- Sazer, S. & Nurse, P. (1994), ‘A fission yeast RCC1-related protein is required for the mitosis to interphase transition’, *EMBO J* **13**(3), 606–615.
- Schneider, C., Kudla, G., Wlotzka, W., Tuck, A. & Tollervy, D. (2012), ‘Transcriptome-wide analysis of exosome targets’, *Mol. Cell.* **48**(3), 422–433.

- Segref, A., Mattaj, I. & Ohno, M. (2001), ‘The evolutionarily conserved region of the U snRNA export mediator PHAX is a novel RNA-binding domain that is essential for U snRNA export’, *RNA* **7**(3), 351–360.
- Segref, A., Sharma, K., Doye, V., Hellwig, A., Huber, J. & Luhrmann, R. (1997), ‘Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)+ RNA and nuclear pores’, *EMBO J* **16**(11), 3256–3271.
- Shah, S., Wittmann, S., Kilchert, C. & Vasiljeva, L. (2014), ‘lncRNA recruits RNAi and the exosome to dynamically regulate pho1 expression in response to phosphate levels in fission yeast’, *Genes Dev.* **28**, 231–244.
- Shichino, Y., Otsubo, Y., Yamamoto, M. & Yamashita, A. (2020), ‘Meiotic gene silencing complex MTREC/NURS recruits the nuclear exosome to YTH-RNA-binding protein Mmi1’, *PloS Genet.* **16**(2).
- Shiomi, T., Fukushima, K., Suzuki, N., Nakashima, N., Noguchi, E. & Nishimoto, T. (1997), ‘Human dis3p, which binds to either GTP- or GDP-Ran, complements *Saccharomyces cerevisiae* Dis3’, *J. Biochem.* **123**(5), 883–890.
- Silla, T., Karadoulama, E., Makosa, D., Lubas, M. & Jensen, T. (2018), ‘The RNA exosome adaptor ZFC3H1 functionally competes with nuclear export activity to retain target transcripts’, *Cell Rep.* **23**(7), 2199–2210.
- Snee, M., Wilson, W., Zhu, Y., Chen, S., Wilson, B., Kseib, C., O’Neal, J., Mahajan, N., Tomasson, M., Arur, S. & Skeath, J. (2016), ‘Collaborative control of cell cycle progression by the RNA exonuclease Dis3 and Ras is conserved across species’, *Genetics* **203**(2), 749–762.
- Sollier, J. & Cimprich, K. (2015), ‘R-loops breaking bad’, *Trends Cell Biol.* **25**(9), 514–522.
- St-Andre, O., Lemieux, C., Perreault, A., Lackner, D., Bahler, J. & Bachand, F. (2010), ‘Negative regulation of meiotic gene expression by the nuclear poly(A)-binding protein in fission yeast’, *J. Biol. Chem.* **285**(36), 27859–27868.
- Sugiyama, T. & Sugioka-Sugiyama, R. (2011), ‘Red1 promotes the elimination of meiosis-specific mRNAs in vegetatively growing fission yeast’, *EMBO J* **30**, 1027–1039.
- Suzuki, N., Noguchi, E., Nakashima, N., Oki, M., Ohba, T., Tartakoff, A., Ohishi, M. & Nishimoto, T. (2001), ‘The *Saccharomyces cerevisiae* small GTPase, Gsp1p/Ran, is

- involved in 3' processing of 7S-to-5.8S rRNA and in degradation of the excised 5'-A0 fragment of 35S pre-rRNA, both of which are carried out by the exosome', *Genetics* **158**(613-625).
- Telekawa, C., Boisvert, F. & Bachand, F. (2018), 'Proteomic profiling and functional characterization of post-translational modifications of the fission yeast RNA exosome', *Nucleic Acids Res.* **46**(21), 11169–11183.
- Thomsen, R., Libri, D., Boulay, J., Rosbash, M. & Jensen, T. (2003), 'Localisation of nuclear retained mRNAs in *Saccharomyces cerevisiae*', *RNA* **9**(9), 1049–1057.
- Torchet, C., Bousquet-Antonelli, C., Milligan, L., Thompson, E., Kufel, J. & Tollervey, D. (2002), 'Processing of 3'-extended read-through transcripts by the exosome can generate functional mRNAs', *Mol. Cell.* **9**, 1285–1296.
- Tudek, A., Schmid, M., Makaras, M., Barrass, J., Beggs, J. & Jensen, T. (2018), 'A nuclear export block triggers the decay of newly synthesized polyadenylated RNA', *Cell Rep.* **24**(9), 2457–2467.
- Van-Hoof, A., Lennertz, P. & Parker, R. (2000), 'Yeast exosome mutants accumulate 3'-extended polyadenylated forms of U4 small nuclear RNA and small nucleolar RNAs', *Mol. Cell. Biol.* **20**(2), 441–452.
- Vanáčová, S., Wolf, J., Martin, G., Blank, D., Dettwiler, S., Friedlein, A., Langen, H., Keith, G. & Keller, W. (2005), 'A new yeast poly(A) polymerase complex involved in RNA quality control', *PLoS Biol.* **3**.
- Vasiljeva, L., Kim, M., Terzi, N., Soares, L. & Buratowski, S. (2008), 'Transcription termination and RNA degradation contribute to silencing of RNA Polymerase II transcription within heterochromatin', *Mol. Cell.* **29**(3), 313–323.
- Vetter, I., Nowak, C., Nishimoto, T., Kuhlmann, J. & Wittinghofer, A. (1999), 'Structure of a Ran-binding domain complexed with Ran bound to a GTP analogue: implications for nuclear transport', *Nature* **398**, 39–46.
- Wan, J., Yourshaw, M., Mamsa, H., Rudnik-Schöneborn, S., Menezes, M., Hong, J., Leong, D., Senderek, J., Salman, M., Chitayat, D., Seeman, P., von Moers, A., Graul-Neumann, L., Kornberg, A., Castro-Gago, M., Sobrido, M., Sanefuji, M., Shieh, P., Salamon, N., Kim, R., Vinters, H., Chen, Z., Zerres, K., Ryan, M., Nelson, S. & Jen, J. (2012),

- ‘Mutations in the RNA exosome component gene EXOSC3 cause pontocerebellar hypoplasia and spinal motor neuron degeneration’, *Nat Genet* **44**(6), 704–708.
- Wang, J., Chen, J., Wu, G., Zhang, H., Du, X. & Chen, S. (2019), ‘NRDE2 negatively regulates exosome functions by inhibiting MTR4 recruitment and exosome interaction’, *Genes Dev.* **33**(9), 526–549.
- Wang, S., Stevenson, A., Kearsey, S., Watt, S. & Bähler, J. (2008), ‘Global role for polyadenylation-assisted nuclear RNA degradation in posttranscriptional gene silencing’, *Mol. Cell. Biol.* **28**(2), 656–665.
- Wasmuth, E. & Lima, C. (2012), ‘Exo- and endoribonucleolytic activities of yeast cytoplasmic and nuclear RNA exosomes are dependent on the noncatalytic core and central channel’, *Mol. Cell.* **48**(1), 133–144.
- Wasmuth, E. V., Januszyk, K. & Lima, C. D. (2014), ‘Structure of an Rrp6-RNA exosome complex bound to poly(A) RNA’, *Nature* **511**, 435–439.
- Wasmuth, E., Zinder, J., Zattas, D., Das, M. & C.D, L. (2017), ‘Structure and reconstitution of yeast Mpp6-nuclear exosome complexes reveals that Mpp6 stimulates RNA decay and recruits the Mtr4 helicase’, *E life* **6**.
- Weick, E., Puno, M., Januszyk, K., Zinder, J., DiMattia, M. & C.D, L. (2018), ‘Helicase-dependent RNA decay illuminated by a cryo-EM structure of a human nuclear RNA exosome-MTR4 complex’, *Cell* **173**(7), 1663–1677.
- Whaba, L., Gore, S. & Koshland, D. (2013), ‘The homologous recombination machinery modulates the formation of RNA-DNA hybrids and associated chromosome instability’, *E life* **2**.
- Wilken, N., Senecal, J., Scheer, U. & Dabauvalle, M. (1995), ‘Localization of the Ran-GTP binding protein RanBP2 at the cytoplasmic side of the nuclear pore complex’, *Eur. J. Cell. Biol.* **68**(3), 211–219.
- Will, C. & Lührmann, R. (2001), ‘Spliceosomal UsnRNP biogenesis, structure and function’, *Curr. Opin. Cell. Biol.* **13**(3), 290–301.
- Win, T., Draper, S., Read, R., Pearce, J., Norbury, C. & Wang, S. (2006), ‘Requirement of fission yeast Cid14 in polyadenylation of rRNAs’, *Mol. Cell.* **26**(5), 1710–1721.

- Wu, H., Becker, D. & Krebber, H. (2014), ‘Telomerase RNA TLC1 shuttling to the cytoplasm requires mRNA export factors and is important for telomere maintenance’, *Cell Rep.* **8**(6), 1630–1638.
- Wyers, F., Rougemaille, M., Badis, G., Séraphin, B., Libri, D. & Jacquier, A. (2005), ‘Cryptic Pol II transcripts are degraded by a nuclear quality control pathway involving a new poly(A) polymerase.’, *Cell* **121**, 725–737.
- Yamanaka, S., Yamashita, A., Harigaya, Y. & Iwata, R. (2010), ‘Importance of polyadenylation in the selective elimination of meiotic mrnas in growing *S. pombe* cells’, *EMBO J* **29**, 2173–2181.
- Yamashita, A., Kariyazono, R. & Watanabe, Y. (2012), ‘Meiotic pairing by non-coding RNA?’, *EMBO J* **13**(9), 766.
- Yamashita, A., Shichino, Y., Tanaka, H., Hiriart, E., Touat-Todeschini, L., Vavasseur, A., Ding, D., Hiraoka, Y., Verdel, A. & Yamamoto, M. (2012), ‘Hexanucleotide motifs mediate recruitment of the RNA elimination machinery to silent meiotic genes’, *Open Biol.* **2**(3).
- Yokoyama, N., Hayashi, N., Seki, T., Pante, N., Ohba, T., Nishii, K., Kuma, K., Hayashida, T., Miyata, T., Aebi, U., Fukui, M. & Nishimoto, T. (1995), ‘A giant nucleopore protein that binds Ran/TC4’, *Nature* **376**, 184–188.
- Zhang, K., Fischer, T., Porter, R., Dhakshnamoorthy, J., Zofall, M., Zhou, M., Veenstra, T. & Grewal, S. (2011), ‘Clr4/Suv39 and RNA quality control factors cooperate to trigger RNAi and suppress antisense RNA’, *Science* **331**(6024), 1624–27.
- Zhou, Y., Zhu, J., Schermann, G., Ohle, C., Bedrin, K. & Sugioka-sugiyama, R. (2015), ‘The fission yeast MTREC complex targets CUTs and unspliced pre-mRNAs to the nuclear exosome’, *Nat. Commun.* **6**(1), 7050.
- Zieve, G., Sauterer, R. & Feeney, R. (1988), ‘Newly synthesized small nuclear RNAs appear transiently in the cytoplasm’, *J. Mol. Biol.* **199**(2), 259–267.