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THÈSE présentée par :

Mateja REMENARIC HAJAK

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Study of ribonucleoprotein particle biogenesis and quality control by a novel technique using bacterial Rho factor as a tool

THÈSE dirigée par :
Dr. A. Rachid RAHMOUNI Directeur de recherche, CNRS Orléans

RAPPORTEURS :
Dr. Lionel MINVIELLE-SEBASTIA Directeur de recherche, INSERM, Bordeaux
Dr. Benoit PALANCADE Chargé de recherche, CNRS, Paris

JURY

Dr. Josette BANROQUES Chargé de recherche, CNRS, Paris
Dr. Lionel MINVIELLE-SEBASTIA Directeur de recherche, INSERM, Bordeaux
Dr. Benoit PALANCADE Chargé de recherche, CNRS, Paris
Pr. Chantal PICHON Professeur d'Université, Orléans – présidente du jury
Dr. A. Rachid RAHMOUNI Directeur de recherche, CNRS, Orléans

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Table of Contents

LIST OF ABBREVIATIONS

7mG	7-methylguanosine
Ala	Alanine
CBC	Cap-binding complex
CE	Capping enzyme
CFIA	Cleavage factor IA
CFIB	Cleavage factor IB
CPF	Cleavage and polyadenylation factors
CTD	Carboxy-terminal domain
CUTs	Cryptic unstable transcripts
DCF	Differential chromatin fractionation
DNA	Deoxyribonucleic acid
eIF4F	Eukaryotic initiation factor 4F
EM	Electron microscopy
FISH	Fluorescent *in situ* hybridization
GTFs	General transcription factors
heptad	Heptapeptide
lncRNA	Long noncoding ribonucleic acid
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
ncRNA	Noncoding ribonucleic acid
NIM	Nrd1 interacting motif
NLS	Nuclear localization signal
NNS	Nrd1-Nab3-Sen1 complex
NPC	Nuclear pore complex
Nrd1C	Nrd1 complex
PAR-CLIP	Photoactivable-ribonucleoside-enhanced UV crosslinking and immunoprecipitation
PAS	Poly(A) signal
PBS	Primary binding site
PCR	Polymerase chain reaction
PIC	Preinitiation complex
Pol I, II, III	Ribonucleic acid polymerase I, II, III
Pro	Proline
Prp19C	Prp19 complex
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RBPs	Ribonucleic acid -binding proteins
REF	Ribonucleic acid and export factor binding protein
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SAXS	Small-Angle X-Ray Scattering
SBS	Secondary binding site
Ser	Serine
snoRNA	Small nucleolar ribonucleic acid
snRNA	Small nuclear ribonucleic acid

TAP	Tandem affinity purification
Thr	Threonine
TRAMP	Trf4/Air2/Mtr4p Polyadenylation complex
tRNA	Transfer ribonucleic acid
Tyr	Tyrosine
UBA	ubiquitin-associated
UTR	untranslated region
wt	wild-type

PREFACE

The research for this thesis was performed in the laboratory group "RNA-proteins interactions and gene regulation" at the Center for Molecular Biophysics (CBM), CNRS Orléans, under supervision of Dr. A. Rachid Rahmouni. It presents new findings and ideas in the field of mRNP biogenesis and quality control in *Saccharomyces cerevisiae*. Thesis was funded with a fellowship from the Ministry of Higher Education and Research of France.

Transcription from DNA into RNA and the biogenesis of a mature messenger RNA particle suitable for export from the nucleus is a highly sophisticated process, far from being completely understood and described. The model developed so far postulates that after emerging from the transcribing polymerase, the nascent transcript is coated with numerous protein factors coupling transcription with mRNA processing events. They also secure transcript integrity, structure and quality for delivering an accurate and adequate message for translation in the cytoplasm. Aberrant transcripts are recognized and quickly degraded by the nuclear degradation machinery in the process of transcript quality control. In recent years the mechanisms of quality control during mRNP biogenesis have been highly researched and discovered for each step of the process. However, due to the complexity of these biogenesis and control events and the abundance of protein factors involved, the dynamic interactions between protein factors themselves and with the transcript still remain elusive.

In an effort to discover and describe new mechanisms of transcription quality control, we developed an innovative technique using a bacterial factor Rho as a tool to produce aberrant transcripts. Rho is a powerful molecular motor capable of removing protein factors off the transcript, thus interfering with the mRNA stability and processing and activating the quality control mechanisms. In our research so far using Rho factor, we have demonstrated a new nuclear quality control recognition mechanism of aberrant transcripts and identified many factors involved in mRNP biogenesis as suppressors of Rho-induced transcript defectiveness. In this thesis I will present additional findings regarding transcript degradation of Rho-induced aberrant transcripts and dissect the influence of Rho expression on a protein complex crucial for proper mRNP biogenesis, thus uncovering its new characteristics and possible roles in coupling transcription to mRNA packaging, processing and export.

1 INTRODUCTION

1.1 mRNP biogenesis is co-transcriptional in *Saccharomyces cerevisiae*

Transcription, although being one of the processes most essential to life, is still far from being completely unraveled. While details of the process may differ among eukaryotes, its universal nature potentiates extensive research using model organisms, among them *Saccharomyces cerevisiae*. The complexity of transcription lies not only in the three major steps of initiation, elongation and termination, but also in the co-transcriptional coupling with maturation, packaging and export of messenger ribonucleic acids (mRNAs).

In eukaryotes synthesis of the ribonucleic acid (RNA) is confined to the cell nucleus and is carried out by three RNA polymerases. All three are structurally similar but are commonly regarded as having distinct roles and properties. RNA polymerase I (Pol I) transcribes the large precursor for 5.8S, 18S and 28S ribosomal RNAs (rRNAs). RNA polymerase II (Pol II) synthesizes all protein coding (mRNAs) and also some noncoding RNAs (ncRNAs), like small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), micro RNA (miRNA), long noncoding RNA (lncRNA), cryptic unstable transcripts (CUTs) and others. RNA polymerase III (Pol III) catalyzes the transcription of other small ncRNAs, amongst them transfer RNA (tRNA) and 5S rRNA. In recent years evidence of common transcription units for both Pol II and III have emerged, which suggests RNA polymerase initiation specificity can vary according to surrounding conditions (Raha et al., 2010; Jamonnak et al., 2011; Duttke, 2014). Regulation of mRNA transcription is dependent upon promoter strength, availability of specific transcription factors and also the state of chromatin structure. Influence of the latter reaches all the way into productiveness of Pol II elongation, the ability to terminate transcription and may even effect mRNA processing (Alén et al., 2002; Morillon et al., 2003; Murawska and Brehm, 2011). In classic general story of mRNA biogenesis, pre-mRNA is firstly transcribed, then protected at both ends by addition of 7-methylguanosine (7mG) cap to the 5'end and polyadenylation (poly(A)) at the 3'end, and finalized by excision of introns and exon ligation. However, in living cells, transcription and most of the processing steps are physically and functionally coupled, thus enhancing the efficiency and accuracy of mRNA maturation. The newly formed transcript is co-transcriptionally assembled with specific protein factors, which ensure the production of a mature, export-competent messenger ribonucleoprotein particle (mRNP).

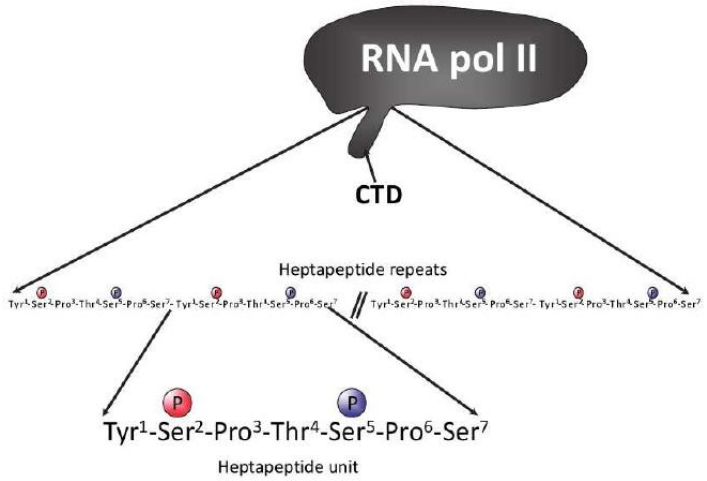
1.1.1 RNA Polymerase II CTD

The steps of mRNA processing, namely capping, splicing and polyadenylation, are tightly coupled to each other and to transcription. Co-transcriptional recruitment of transcription and processing factors is coordinated by the carboxy-terminal domain (CTD) of the largest subunit of Pol II.

The CTD of Pol II (reviewed in Corden, 2013; Eick and Geyer, 2013) is an unusual and unique, largely flexible domain (Meinhart et al., 2005). It comprises multiple heptapeptide (heptad) repeats, containing 4 different amino acids: serine (Ser), proline (Pro), tyrosine (Tyr) and threonine (Thr), with the consensus sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 ($Y_1S_2P_3T_4S_5P_6S_7$) (Figure 1.1A) (Hall and Georgel, 2011). The CTD consensus sequence is conserved in *S. cerevisiae* and mammals, yet it differs markedly in its length with 26 and 52 repeats, respectively. The heptads are said to come in tandems, as the insertion of an alanine (Ala) residue between two heptads in yeast is lethal, while the same insertion between two diheptads is tolerable, therefore it was believed to be a functional unit of the yeast CTD (Stiller and Cook, 2004). More recently, even smaller functional unit was defined, containing only the first 11 residues of a diheptad where the most essential are three serine-proline motifs and two tyrosine residues spaced at a heptad interval (Liu et al., 2010) (Figure 1.1B). However, the same study reveals that CTD length is more crucial for viability than the number of functional units contained within the amino acid sequence. For *S. cerevisiae*, minimal viable CTD length is 8 heptads containing 7 functional units (West and Corden, 1995).

Recruitment of different factors during transcription is achieved by extensive post-translational and conformational modifications of the CTD heptad repeats. These include phosphorylation of Tyr, Thr and Ser, glycosylation of Thr and Ser, and isomerization of Pro residues. Additional modifications are possible in non-consensus heptads at the end of mammalian CTD (Chapman et al., 2005; Napolitano et al., 2014), expanding even more the number of combinatorial possibilities orchestrating protein association and dissociation and defining the CTD code (Buratowski, 2003; Cassart et al., 2012; Schwer et al., 2014; reviewed in Egloff et al., 2012) (Figure 1.1C).

A



B

Diheptad

Y^1-S^2-P^3-T^4-S^5-P^6-S^7-Y^1-S^2-P^3-T^4-S^5-P^6-S^7

Minimal functional unit

Y^1-S^2-P^3-$(X)^4$-S^5-P^6-$(X)^7$-Y^8-S^9-P^{10}-$(X)^{11}$

C

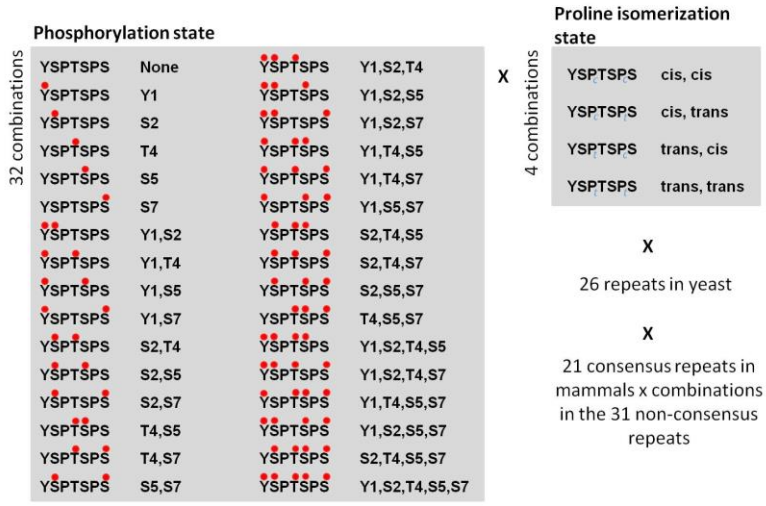


Figure 1.1: *CTD modifications orchestrate binding and exchange of protein factors and complexes involved in mRNA processing*. (A) Heptapeptide consensus repeats of the CTD of the Pol II large subunit (Hall and Georgel, 2011). (B) CTD functional units: a diheptad proposed by Stiller and Cook (2004); a minimal functional unit determined by Liu et al. (2010). (C) Potential phosphorylation and proline isomerization combinations for CTD modification during transcription (adapted from Egloff et al., 2012). Phosphorylation is indicated by red circles and trans or cis isomerization of prolines by a blue t or c, respectively, below the amino acid.

Among distinct CTD modifications, phosphorylation is the best characterized and is crucial in pre-mRNA maturation. The phosphorylation of Ser2 and Ser5 residues in a heptad sequence was firstly discovered more than 20 years ago (Zhang and Corden, 1991). It has remained in the focus of interest for the most part of that period, until the discovery of an *in vivo* Ser7 phosphorylation by Chapman et al., 2007. Although the potential for phosphorylation of Tyr and Thr residues in mammalian cells was described simultaneously as Ser2/5 phosphorylation (Zhang and Corden, 1991; Baskaran et al., 1993), their phosphorylation in the consensus sequence, in yeast, as well as their functions were discovered only recently (Sakurai and Ishihama, 2002; Hsin et al., 2011; Mayer et al., 2012), thus extending the CTD code.

1.1.2 Co-transcriptional mRNA processing

Phosphorylation code of the CTD has begun to be depicted in more detail (Figure 1.2) with the use of monoclonal antibodies in chromatin immunoprecipitation (ChIP) experiments (reviewed in Heidemann et al., 2013). They represent a powerful tool to study CTD modification patterns due to their specific recognition of the unphosphorylated CTD and the single or double phosphorylation marks (Ser-, Tyr-, Thr- or Ser2/Ser5) (Heidemann et al., 2013). Each antibody recognizes not only the phosphorylated mark but also the adjacent amino acids, which minimizes the recognition of the same phosphorylated amino acid residues on other proteins (Eick and Geyer, 2013).

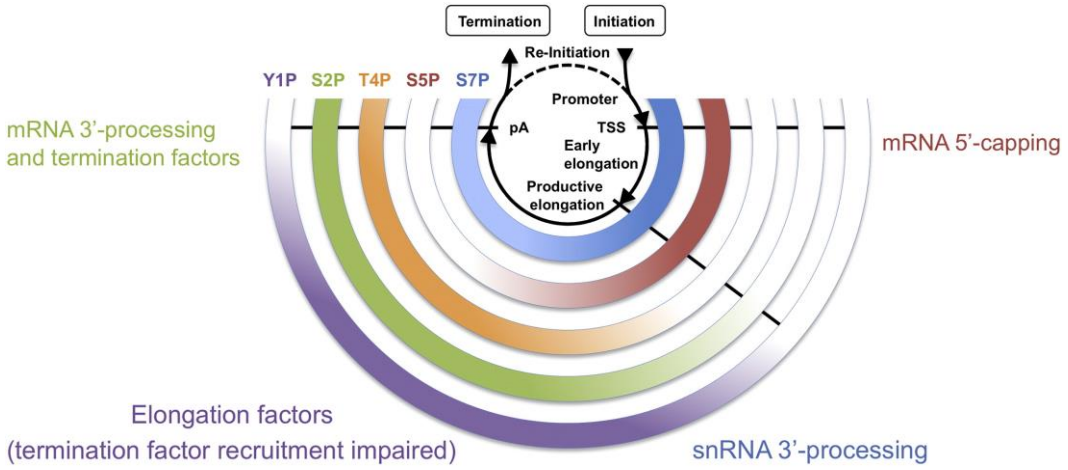


Figure 1.2: *Schematic representation of the averaged CTD code profile, obtained by ChIP experiments* (Heidemann et al., 2013). Color gradients illustrate changing phosphorylation levels of Tyr1 (violet), Ser2 (green), Thr4 (orange), Ser5 (red) and Ser7 (blue) residues during different steps of transcription. The color of each noted transcription step corresponds to the color of the implicated phosphorylation mark. Ser5-P facilitates promoter escape and recruitment of the capping enzyme (CE) (see page 10). Ser7-P is involved in recruiting the Integrator complex in the 3'end processing pathway of snRNA-encoding genes in mammals, while its role in protein coding genes has not yet been determined (see pages 11 and 16). Tyr1 is phosphorylated in the middle of the transcription process and it seems to act in discriminating between elongation and termination since it impairs the recruitment of transcription termination factors (see page 16). Phosphorylation of Ser2 marks the beginning of the elongation phase. The level of Ser2-P increases during transcription and recruits many processing factors, among them 3'end processing and termination factors at the end of transcription (see pages 11 and 12).

Even though the use of monoclonal antibodies is common nowadays, the shortcomings of this technique, such as concealed phospho-epitopes and cross-reactivity, impede the unambiguous interpretation of the obtained data (Eick and Geyer, 2013). Future perspectives place hope in the use of mass spectrometry analysis in the research of CTD modifications. Nonetheless, ChIP results have led to important discoveries of CTD phosphorylation patterns, phosphorylation and dephosphorylation agents, as well as protein factor recruitment at distinct time-points during transcription corresponding to different phosphorylation marks (Figure 1.3).

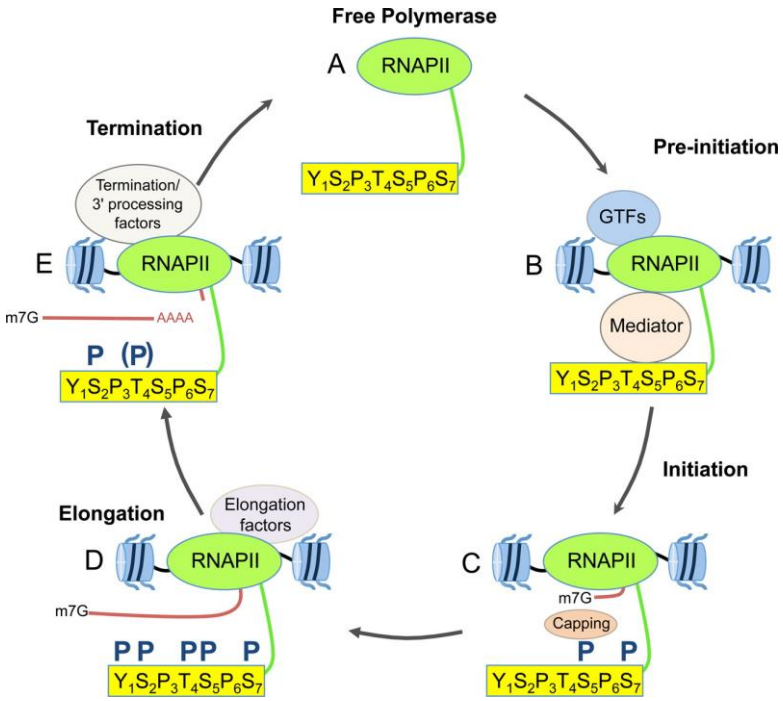


Figure 1.3: *Phosphorylation states and factors recruited during Pol II transcription cycle* (Eick and Geyer, 2013). (A) Free polymerase in a hypo-phosphorylated state. (B) General transcription factors (GTFs) recruit Pol II and the Mediator complex to the promoter, thus forming the preinitiation complex (PIC). (C) Ser5-P marks the intiation phase and recruits capping enzymes. (D) Dynamic phosphorylation and de-phosphorylation of the CTD during elongation phase recruits many elongation and processing factors. (E) Programming of the CTD for termination, gradual removal of CTD phosphorylation marks by phosphatases, and release of Pol II and transcripts from the template.

1.1.2.1 Coupling transcription with mRNA capping and splicing

At the beginning of a transcription cycle, Pol II with hypo-phosphorylated CTD is recruited to promoter by general transcription factors (GTFs), along with gene-specific transcription factors and the Mediator complex, forming the preinitiation complex (PIC) (Zhang et al., 2012a; Murakami et al., 2013) (Figure 1.3B). Transcription passes into initiation phase with first CTD phosphorylation at Ser5 position by the Kin28 kinase, a component of general transcription factor TFIIH (Komarnitsky et al., 2000). This modification endorses promoter escape and stimulates dissociation of the Mediator complex (Wong et al., 2014). Another yeast kinase, Srb10 (homolog of CDK8 in mammals), has also been shown to phosphorylate CTD at Ser5 positions *in vivo*, but its actual contribution is still

unclear (Galbraith et al., 2010). Likewise, phosphorylation of Ser5 (Ser5-P) promotes recruitment of RNA capping enzyme (CE), which protects the 5'end of the nascent transcript from degradation by adding a 7mG cap at the 5'end (Suh et al., 2010) (Figure 1.3C). In eukaryotes, capping is a three step enzymatic process: (i) the triphosphate 5' end of the nascent transcript is hydrolyzed to a diphosphate; (ii) a guanosine monophosphate (GMP) is transferred to the diphosphate in a 5'-5' linkage, forming a GpppN structure which is (iii) finally methylated, thus forming a complete 7mG cap (Schwer et al., 2000). The first two steps in yeast are performed by the CE made out of two tightly associated enzymes, an RNA triphosphatase (Cet1p) and a guanylyltransferase (Ceg1p), while in higher eukaryotes these two enzymatic functions are combined in a single bifunctional protein (Takase et al., 2000). The final step is carried out by a methyltransferase (Abd1 in yeast). The mature cap structure is then associated with other complexes, such as cap-binding complex (CBC) in the nucleus and eukaryotic initiation factor 4F (eIF4F) in the cytoplasm, which mediate further transcript processing, export and translation (Gonatopoulos-Pournatzis and Cowling, 2014).

The general elongation complex displaces PIC after successful promoter escape and Ser2 phosphorylation (Ser2-P) marks the beginning of the elongation phase (Mayer et al., 2010) (Figure 1.3D). Ser5-P recruits Bur1 kinase, which performs the initial Ser2 phosphorylation, while more extensive Ser2 phosphorylation is carried out by Ctk1, a catalytic subunit of CTD kinase-I, thus promoting transition into processive elongation (Jones et al., 2004; Bataille et al., 2012). Ser7 phosphorylation is suggested to be another signal of the CTD code important for promoting transcription elongation (Czudnochowski et al., 2012). This mark is set by Kin28 kinase at the promoter, but is maintained along the coding region by Bur1 kinase and is at high level until the very 3'end (Chapman et al., 2007; Tietjen et al., 2010). Nevertheless, the role of Ser7-P in transcription elongation has yet to be further investigated and confirmed.

Concurrent with the beginning of elongation, starts the removal of Ser5-P marks by Rtr1 phosphatase (Mosley et al., 2009). Consequent change in the ratio of Ser2/Ser5 phosphorylation signals the recruitment of processing complexes, which have yet to perform their functions. First in line is spliceosome, one of the largest macromolecular complexes found in living cells. It is composed of a minimum of ~100 proteins associated with five snRNAs (Hoskins et al., 2011). CTD has been shown to bind several splicing factors and they can sequentially recruit additional splicing machinery, i.e. the spliceosome activating Prp19

complex (Prp19C). This complex is also able to enhance transcription elongation (Hsin and Manley, 2012). Aside from its interaction with the CTD, splicing has been shown to be coupled with transcription by CBC dependent spliceosome assembly (Görnemann et al., 2005), it highly interacts with 3'end processing machinery in a reciprocal co-regulation (Li et al., 2001; Rigo and Martinson, 2009) and spliceosome could even be involved in regulation of gene expression (Volanakis et al., 2013).

1.1.2.2 Coupling transcription with 3' end processing

As for splicing, phosphorylation of Ser2 residue is also important for 3'end processing events (Richard and Manley, 2009) (Figure 3E). These events can be carried out in three distinct pathways, depending on the type of RNA being transcribed: pre-mRNAs in poly(A)-dependent pathway, snoRNAs and CUTs in poly(A)-independent pathway and snRNAs in Integrator-dependent processing-termination pathway (Eick and Geyer, 2013).

1.1.2.2.1 Polyadenylation-dependent 3' end processing

The first pathway for 3'end processing is polyadenylation-dependent, thus it demands the presence of a specific poly(A) signal (PAS) in the nascent RNA, which is recognized by cleavage factors IA and IB (CFIA, CFIB), and cleavage and polyadenylation factors (CPF) (Dichtl and Keller, 2001; Mischo and Proudfoot, 2013) (Figure 1.4). Several representatives of these complexes have been shown to interact with phosphorylated CTD, a characteristic not essential for 3'end processing, but largely enhancing its efficiency (Rigo et al., 2005; Kuehner et al., 2011).

CFIA is a multisubunit complex which associates with Pol II and functions by selecting the cleavage site and assisting the recruitment of polyadenylation factors. Thus, 3'end formation is a two-step process: pre-mRNA is first cleaved and subsequently polyadenylated for protection from 3' to 5'end exonucleolysis (Zhao et al., 1999). The cleavage and polyadenylation factor Pcf11, one of the CFIA members, contains a CTD Interacting Domain (CID) which preferentially interacts with Ser2-P (Barillà et al., 2001; Lunde et al., 2010; Gu et al., 2013). However, Pcf11-CID likewise binds the nascent RNA. This dual binding capability reflects double function of this factor during transcription. In addition to cleavage and polyadenylation, Pcf11 plays a role in transcription termination,

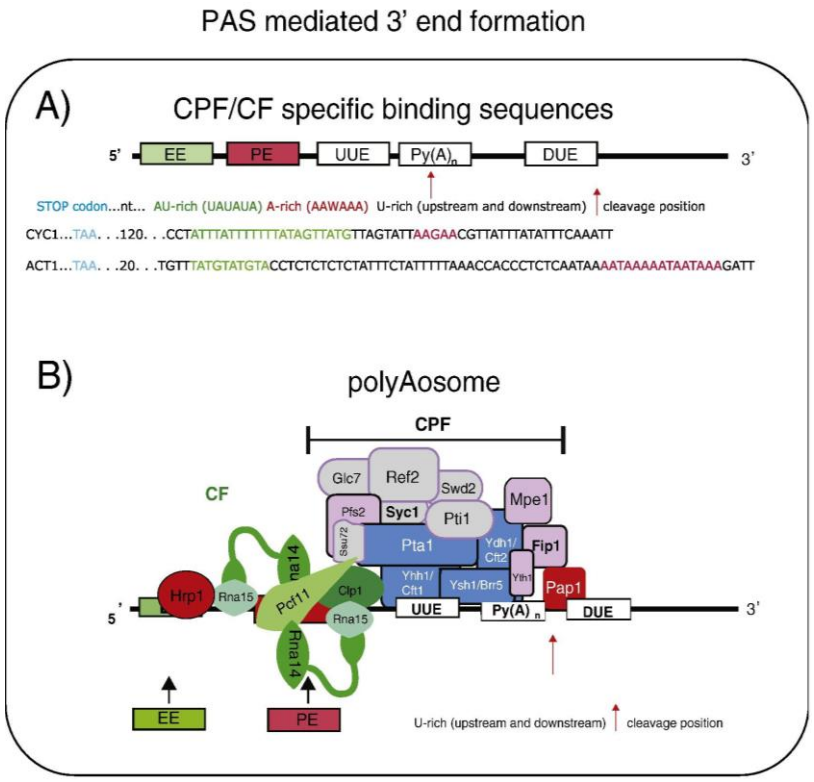


Figure 1.4: *Polyadenylation-dependant 3'end processing signals and protein factors in yeast* (adapted from Mischo and Proudfoot, 2013). (A) CPF/CF specific binding sequences comprise the AU-rich efficiency element (EE), the A-rich positioning element (PE) and U-rich regions preceding the cleavage site. Low conservation of different sequence elements is exemplified by polyadenylation signals of CYC1 and ACT1 mRNAs. (B) Composition and suggested organisation of the CPF/CF complex on mRNAs. CPF members are represented in blue, pink and gray and CF members are in green and red.

together with the termination factor Rtt103 (Sadowski et al., 2003; Zhang, 2005; Hollingworth et al., 2006).

Table 1.1: Factors involved in Pol II 3'end processing and/or transcription termination (Kuehner et al., 2011).

Yeast complex	Yeast protein	Human homologue?	Protein function	Putative role in termination	
				Poly(A)-dependent	Sen1-dependent
CFIA	Pcf11	PCF11	Binds Pol II Ser2-P CTD, scaffolding protein	Promotes RNA cleavage and Rat1 recruitment, disrupts Pol II hybrid by bridging CTD and RNA	Disrupts Pol II hybrid by bridging CTD and RNA
CPF	Cft1	CPSF160	Binds Pol II Ser5-P and Ser2-P CTD, binds poly(A) site RNA	Promotes Pol II pausing and RNA cleavage for Rat1 entry	N.D.
CPF	Ysh1	CPSF73	Endoribonuclease, cleaves poly(A) site RNA	Provides entry point for Rat1	Provides entry point for exoribonuclease‡
CPF	Yth1	CPSF30	Binds poly(A) site RNA and Pol II	Promotes Pol II pausing	N.D.
CPF(APT§)	Glc7	PP1	Ser/Thr phosphatase, dephosphorylates Sen1	N.D.	Promotes Sen1 recruitment and/or helicase activity
CPF (APT)	Pta1	Symplekin	Scaffolding protein, bridges CFIA and CPF	N.D.	Maintains integrity of APT complex
CPF (APT)	Ssu72	SSU72	Pol II Ser5-P CTD phosphatase	Promotes recruitment of Pcf11 to Pol II‖	Promotes recruitment of Pcf11 to Pol II
Rat1–Rai1–Rtt103	Rat1	XRN2	5′-3′ exoribonuclease, degrades Ysh1 (or CPSF73)-generated downstream cleavage product	Promotes Pcf11 and Rna15 recruitment, collides with Pol II near RNA exit channel	No effect observed
Rat1–Rai1–Rtt103	Rai1	DOM3Z	De-capping endoribonuclease, pyrophosphohydrolase	Promotes Rat1 stability and activity	N.D.
Rat1–Rai1–Rtt103	Rtt103	–	Binds Pol II Ser2-P CTD, bridges Rat1 to Pol II CTD	Recruits Rat1 and Pcf11 to Pol II	N.D.
–	–	p54NRB and PSF	RNA-binding proteins, bind Pol II CTD	Promotes XRN2 recruitment to Pol II	N.D.
Sen1–Nrd1–Nab3	Nrd1	SCAF8 and SCAF4	Binds Pol II Ser5-P CTD, RNA-binding protein	No effect observed	Disrupts Pol II hybrid by bridging CTD and RNA, recruits Sen1 to Pol II
Sen1–Nrd1–Nab3	Nab3	–	RNA-binding protein, bridges Nrd1 and Sen1	N.D.	Recruits Sen1 to Pol II
Sen1–Nrd1–Nab3	Sen1	Senataxin	Binds Pol II CTD, 5′-3′ RNA–DNA helicase	Promotes Rat1 activity by exposing RNA	Unwinds RNA–DNA hybrid in Pol II
Pol II	Rpb1	RPB1	CTD serves as docking site for transcription and RNA processing factors	Recruits Pcf11 and Rat1 to Pol II	Recruits Nrd1, Pcf11 and Sen1 to Pol II
Pol II	Rpb3	RPB3	Forms heterodimer with Rpb11	N.D.	Transduces termination signal to Pol II
Pol II	Rpb11	RPB11	Forms heterodimer with Rpb3	N.D.	Transduces termination signal to Pol II
Paf1C	Paf1	PAF1	Associates with Pol II, scaffolding protein	N.D.	Promotes recruitment of Pcf11 and Nrd1 to Pol II
–	Ess1	PIN1	Prolyl-isomerase; binds Ser5-P and Pro_6 Pol II CTD	Promotes recruitment of Ssu72 to Pol II‖	Promotes recruitment of Ssu72 to Pol II
–	Ctk1	CDK9	Pol II Ser_2 CTD kinase	Promotes recruitment of CFIA and CPF¶	Promotes recruitment of Pcf11
–	Chd1	CHD1	Chromatin-remodelling factor	Enhances Pol II pausing	N.D.

APT, associated with Pta1; CDK9, cyclin-dependent kinase 9; CFIA, cleavage factor IA; Chd1, chromodomain helicase DNA-binding 1; CPF, cleavage and polyadenylation factor; CPSF, cleavage and polyadenylation specificity factor; CTD, carboxy-terminal domain; Ctk1, CTD kinase subunit 1; DOM3Z, DOM-3 homologue Z; N.D., not determined; Nab3, nuclear polyadenylated RNA-binding 3; Paf1C, Paf1 complex; Pcf11, protein 1 of CFI; PIN1, peptidyl-prolyl *cis–trans* isomerase NIMA-interacting 1; Pol II, RNA polymerase II; PP1, protein phosphatase 1; Rai1, Rat1-interacting 1; Rat1, RNA-trafficking protein 1; Rtt103, regulator of Ty1 transposition 103; SCAF, SR-related and CTD-associated factor; Ssu72, suppressor of Sua7 2; XRN2, 5′–3′ exoribonuclease 2; *This is an abbreviated listing owing to space constraints. A more complete table is included in Supplementary information S1 (table). ‡Termination defects are allele-specific. §APT is a subcomplex of yeast CPF. ‖Role in termination seems to be limited to specific mRNAs (for example, short genes and targets of Sen1-dependent attenuation). ¶Role in termination seems to be limited to mRNAs with weak poly(A) sites.

Transcription termination is tightly connected to 3'end processing. It is hard to distinguish precisely where one begins and the other finishes, since many factors are involved in both processes (Table 1.1). Key event in termination is Pol II disengagement from the deoxyribonucleic acid (DNA) template. At first, two models were proposed to explain transcription termination of protein coding genes: the allosteric or anti-terminator model implies that transcription through PAS leads to a conformational change of elongation complex, while the torpedo model suggests that cleavage at the poly(A) site enables the 5'-3' exonuclease Rat1 to load onto the unprotected 5'end of the RNA left emerging from the polymerase after the cut. Rat1 degrades the RNA and, in some still undefined way, promotes Pol II release after "catching up" with it (Richard and Manley, 2009). Lately, a unified model has mostly been advocated, since Rat1 action was shown insufficient for Pol II release from the template. Nevertheless, the main actor responsible for Pol II disengagement remains obscure (Luo et al., 2006; Richard and Manley, 2009; Schaughency et al., 2014).

1.1.2.2.2 Polyadenylation-independent 3' end processing

The second 3'end processing and/or termination pathway is poly(A)-independent and directed by the Nrd1 complex (Nrd1C or Nrd1-Nab3-Sen1 complex (NNS)), which consists of RNA binding proteins Nrd1 and Nab3, and the RNA helicase Sen1 (Steinmetz et al., 2001) (also called Sen1-dependent termination, see Table 1.1). This complex interacts with CTD through Nrd1-CID, which preferentially binds at Ser5-P, thus explaining the Nrd1 crosslinking near 5'end of non-coding and coding genes, and it's role in termination of short Pol II-transcribed genes (Vasiljeva et al., 2008; Kubicek et al., 2012). Sen1 also binds CTD but interacts with Ser2-P along the whole length of both non-coding and coding genes, and it was proposed to terminate transcription in a way similar to bacterial Rho helicase; this factor shall be presented in section 1.3 (Steinmetz et al., 2006; Creamer et al., 2011; Kuehner et al., 2011; Chinchilla et al., 2012; Porrua and Libri, 2013a). Nrd1 was originally believed to have a specific sequence binding site. However, its binding affinity has now been extended to AU-rich, GU-rich and G-rich sequences, thus extending the pool of possible RNA substrates targeted by Nrd1C (Creamer et al., 2011; Bacikova et al., 2014). Nevertheless, for functional termination by Nrd1C the mere presence of binding sequences is not enough. Their arrangement and association in supermotifs is crucial for termination (Porrua et al., 2012). Indeed, this is in accordance with recent findings of Nrd1C function in processing of even

mRNA coding genes and other types of RNAs. This function is mostly connected with nutrient response, suggesting a possible overlap of termination pathways which were before believed distinct (Jamonnak et al., 2011; Darby et al., 2012; Webb et al., 2014). Another interaction of Nrd1C is realized with the exosome, which leads to 3'end trimming or transcript degradation in a quality control process, which will also be described in more details later on (section 1.2.2.2) (Vasiljeva and Buratowski, 2006).

Another part of the CTD code is Tyr1 phosphorylation. The replacement of Tyr1 residue was proven to be lethal in yeast (West and Corden, 1995; Schwer and Shuman, 2011). This mark seems to aid in the discrimination between elongation and transcription termination, by preventing interaction of early (Nrd1) and late (Pcf11, Rtt103) termination factors with the CTD. Thus, Tyr1 is phosphorylated during transcription of the central region of genes (Figure 1.2), yet its presence does not impair CTD binding of the elongation factor Spt6 (Mayer et al., 2012). Although yeast Tyr1 kinase has not yet been found, very recently a CPF subunit Glc7 was shown to dephosphorylate Tyr1 *in vitro* and *in vivo* in *S. cerevisiae*. This finding, along with Glc7 role in recruiting termination factors Pcf11 and Rtt103, provides yet another connection between 3'end processing and termination (Schreieck et al., 2014).

1.1.2.2.3 Integrator-dependent 3' end processing

Finally, the third 3'end processing pathway relies on Ser2 and Ser7 phosphorylation recruiting the Integrator complex to snRNA-encoding genes in mammals, while yeast homologs of this complex's subunits have not been found (Baillat et al., 2005; Egloff et al., 2010). Contrary to the initial idea of a universal, genome-wide CTD code, Ser7 phosphorylation was first discovered as gene-specific, required for snRNA gene expression (Corden, 2007; Egloff et al., 2007). Thr4 phosphorylation was the next one, shown to be required for histone mRNA 3'end processing in chicken cells, and most recently, it was revealed as a regulator of expression of specific genes in *S. cerevisiae* (Hsin et al., 2011; Rosonina et al., 2014). Nevertheless, genome-wide studies performed until now have presented biased results regarding uniformity and/or specificity of CTD phosphorylation patterns, accentuating the need for further examination (Kim et al., 2010; Mayer et al., 2010; Tietjen et al., 2010; Bataille et al., 2012).

After successful transcription termination, by any of the three described pathways, Pol II CTD returns into its hypo-phosphorylated state (Figure 1.3A). Serine phosphatases have been best described so far (Figure 1.5), while a Thr4 phosphatase has not yet been determined. Ser5-P and Ser7-P are coupled by Kin28 phosphorylation at the beginning and Ssu72 de-phosphorylation at the end of transcription, while Ser5 phosphorylation is additionally removed from the start of elongation by an ill-defined Rtr1 phosphatase (Bataille et al., 2012), mentioned earlier (section 1.1.2.1). Ser2 phosphatase Fcp1 travels with Pol II and interplays with Ser2 kinase Ctk1, thus dynamically regulating Ser2 phosphorylation level (Cho et al., 2001). The same phosphatase performs complete Ser2-P de-phosphorylation after transcription termination (Bataille et al., 2012). In addition to the aforementioned importance of Tyr1 de-phosphorylation in transcription termination, the same was recently presented for Ser7 mark (Zhang et al., 2012b). The complete CTD de-phosphorylation allows the Pol II to be recycled and start another round of transcription (Cho et al., 1999).

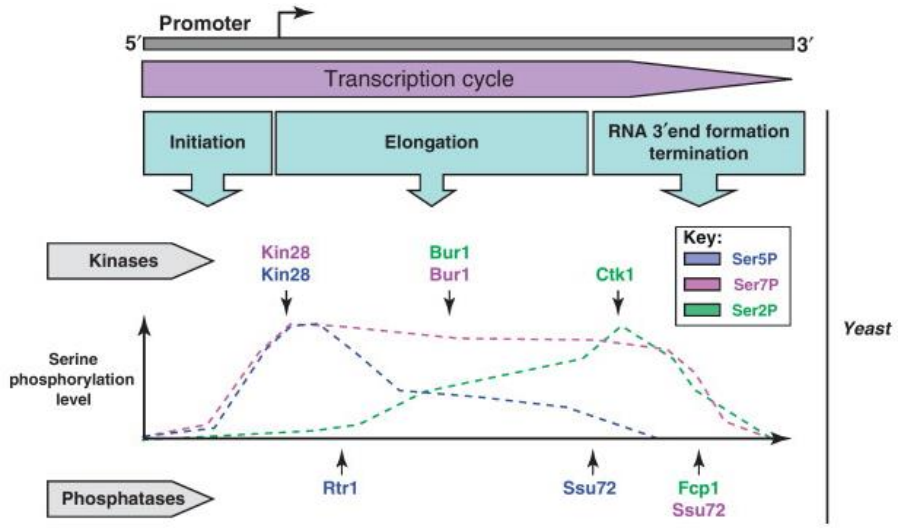


Figure 1.5: *Phosphorylation patterns of the Pol II CTD Ser marks during transcription cycle of protein coding genes in yeast* (Egloff et al., 2012). Characterized kinases and phosphatases which establish this patterns are represented in colors annotated to each Ser-P mark on which they act.

1.1.3 Co-transcriptional mRNP assembly coupled with export

mRNA has an important role of transferring information for protein synthesis from the coding DNA in the nucleus to ribosomal machinery in the cytoplasm. For production of a functional protein the newly formed transcript in the cell nucleus has to be properly processed and packaged with different proteins ensuring its integrity and directing it to export through the nuclear pore complex (NPC) (Aguilera, 2005; Luna et al., 2008) (Figure 1.6).

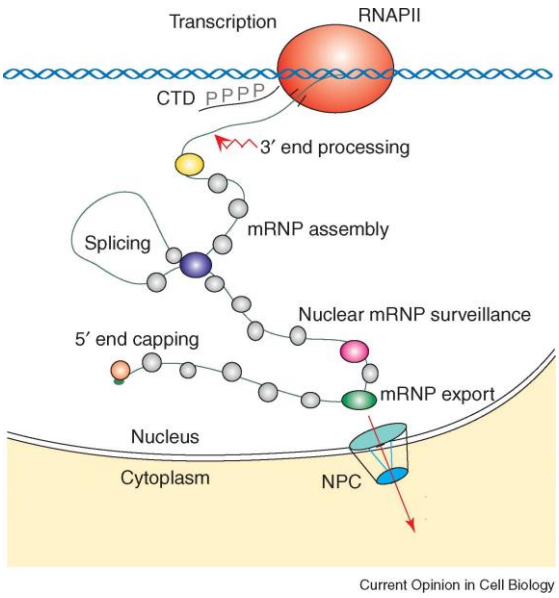


Figure 1.6: *Simplified model of mRNP biogenesis steps producing export-competent transcripts* (Aguilera, 2005).

Nascent transcript is promptly associated with diverse protein factors as it emerges from the transcription machinery. Composition of this mRNP complex is not fixed but highly dynamic and interactive, as presented for processing factors in previous subchapter. Aside from members of the core transcription and processing machineries, mRNP is formed by many RNA-binding proteins (RBPs) and other proteins associated via protein-protein interactions (Müller-McNicoll and Neugebauer, 2013; Mitchell and Parker, 2014). Photoactivable-ribonucleoside-enhanced UV crosslinking and immunoprecipitation (PAR-CLIP) performed in human and yeast cells was used to identify ~800 and ~120 RBPs bound to a mature mRNA, respectively (Baltz et al., 2012; Mitchell et al., 2013). RBPs have an important role in proper packaging of mRNA to prevent excessive interactions between

nascent RNA and template DNA, which can lead to RNA:DNA hybrid formation. Hence, RPBs function in preventing genomic instability and at the same time they have to ensure enough flexibility to allow efficient processing steps (Müller-McNicoll and Neugebauer, 2013; Hamperl and Cimprich, 2014). After the release from transcription site, mRNP has to be perfectly compacted for diffusion through the nucleoplasm and towards the nuclear periphery (Mor et al., 2010; Oeffinger and Zenklusen, 2012). Electron microscopy (EM) analysis showed that mRNPs purified from budding yeast have an elongated, ribbon-like shape with lateral constrictions, are 5-7 nm thick and with length of 20-30 nm, increasing proportionally with the mRNA length (Batisse et al., 2009) (Figure 1.7).

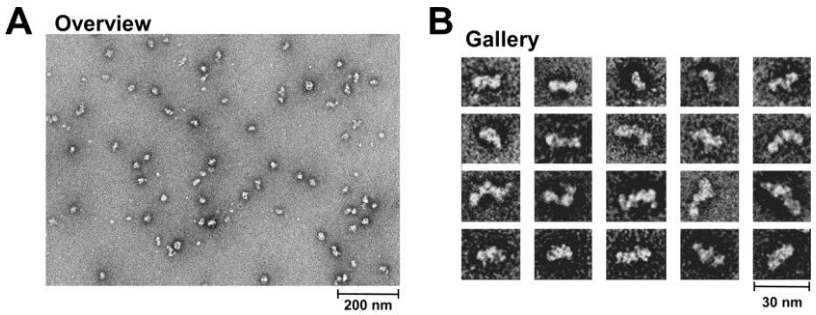


Figure 1.7: *Electron microscopy analysis of yeast mRNPs* (Batisse et al., 2009). Visualization of mRNPs which were affinity purified by tandem affinity purification (TAP) tagged Nab2 factor. (A) An overview of one fraction from sucrose gradient. (B) A gallery of single mRNP particles.

In yeast, key components of this mRNP packaging process are the CTD of Pol II and a protein complex named THO, both implicated in recruitment of majority of RPBs and other mRNP protein factors (Tutucci and Stutz, 2011; Katahira, 2012; Oeffinger and Zenklusen, 2012). THO travels along with Pol II during transcription elongation and co-transcriptionally recruits mRNA export factors Yra1 and Sub2 in stoichiometric quantities (Strasser et al., 2002). Together they form TREX complex (TRanscription/EXport), which is conserved from yeast to humans and is believed indispensable for connection between transcription, mRNP assembly and export (Aguilera, 2005; Katahira, 2012).

1.1.3.1 THO complex

In *Saccharomyces cerevisiae* THO complex is characterized as a heteropentameric assembly, composed of Tho2 (184 kDa), Hpr1 (88 kDa), Tex1 (47 kDa), Mft1 (45 kDa) and Thp2 (33 kDa) (Chavez et al., 2000; Strasser et al., 2002). Recent studies with a focus on solving THO complex architecture confirm its five subunits structure (Pena et al., 2012; Poulsen et al., 2014) (Figure 1.8). However, not all members demonstrate the same relevance to THO complex integrity and function. In single-subunit THO-null mutants, the complex itself is destabilized and dissociated, with plausible subsequent degradation of other subunits, although mutant strains do not display a major growth defect below 37˚C. (Libri et al., 2002; Huertas et al., 2006).

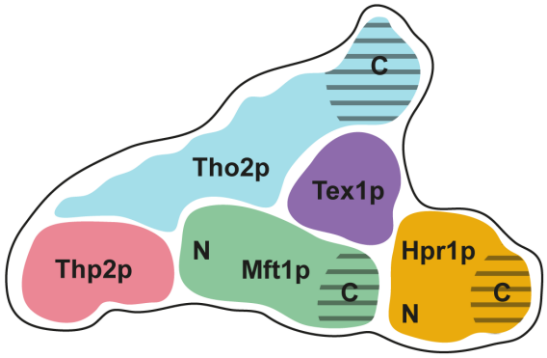


Figure 1.8: *Model of the architectural organization of THO complex* (Poulsen et al., 2014). This pentameric structure of THO complex is based on the combined EM and Small-Angle X-Ray Scattering (SAXS) data. Striped areas indicate predicted flexible areas.

1.1.3.1.1 Transcription site recruitment

As mentioned earlier, THO complex has been shown to travel with Pol II during transcription elongation. Still, the nature of this interaction has remained unresolved for a long time, just like the mechanism of THO recruitment to the transcribed genes (Strasser et al., 2002; Oeffinger and Zenklusen, 2012). Interaction with the aforementioned Prp19C splicing factor ensures THO occupancy on transcribed genes, but only at the 3’ end, whereas the upstream, initial recruitment is independent of this splicing associated complex (Chanarat

et al., 2011). Recently, the highly disordered C-terminal region of Tho2 was characterized as the nucleic acid interacting domain, which facilitates but is not responsible for THO recruitment to chromatin (Pena et al., 2012).

Finally, a novel study by Meinel et al. (2013) revealed that THO is recruited through direct binding to the phosphorylated Pol II CTD. THO complex exhibits the strongest interaction with the Ser2/Ser5 diphosphorylated CTD *in vitro*, while *in vivo* THO recruitment was shown to be dependent on Ser2-P and/or Tyr1-P. However, Ser2 and Tyr1 phosphorylations are most probably interdependent *in vivo*, and by mutating either, it is not possible to precisely determine which one is crucial for THO occupancy. Since THO does not bind Tyr1 phosphorylated CTD *in vitro*, and sn/snoRNA genes are low in both Ser2-P and THO occupancy but high in Tyr1P, one can suppose that Ser2P is the essential mark. Furthermore, THO complex presence increases from 5' to 3' end of a gene, as does Ser2-P, and the Ser2/Ser5 phosphorylation ratio of the CTD is presumed to be the molecular basis for this recruitment pattern (Figure 1.9) (Meinel et al., 2013; Katahira, 2015).

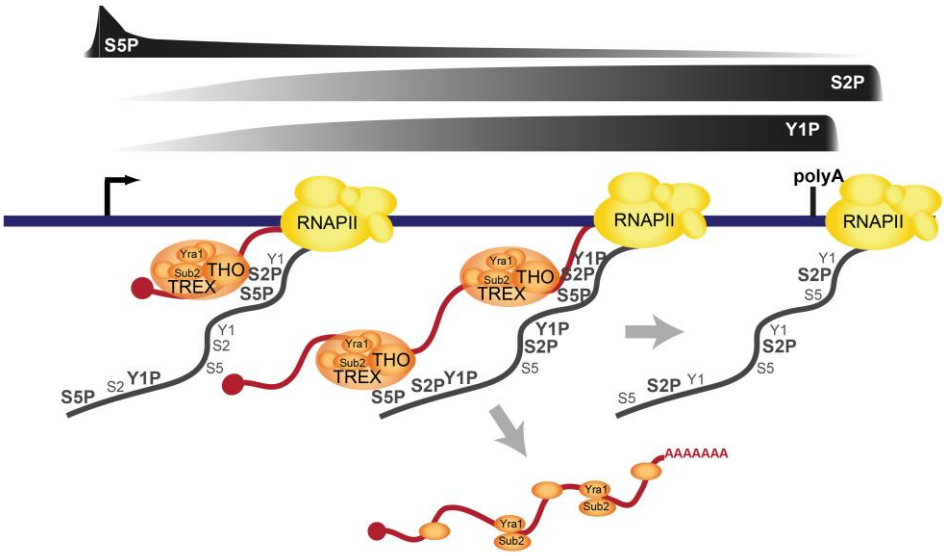


Figure 1.9: *Model of TREX recruitment to transcription site* (Meinel et al., 2013). TREX recruitment is dependent on Ser2/Ser5 phosphorylation ratio and is increased towards the 3' end as the Ser2 phosphorylation is increased. Soon after transcription termination, TREX complex dissociates from the transcription site and no chromatin recruitment can be observed. However, TREX members can still stay bound to the transcript during the process of export.

THO complex 5’ to 3’ increasing occupancy at transcribed genes is a distinctive characteristic differing this complex from other known transcription elongation factors and proteins interacting with the Ser2-P CTD mark (Abruzzi et al., 2004; Gomez-Gonzalez et al., 2011; Meinel et al., 2013). Since THO was shown to bind both DNA and RNA *in vitro* (Jimeno et al., 2002; Pena et al., 2012) the possibility that the observed increase in recruitment is due to THO binding along the nascent RNA has been investigated. ChIP assay coupled with RNase treatment demonstrates that THO chromatin recruitment does not depend on RNA binding (Abruzzi et al., 2004; Pena et al., 2012), while analysis of affinity-purified mRNPs show THO presence on the poly(A) mRNA (Batisse et al., 2009; Bretes et al., 2014). Aforementioned Meinel et al. (2013) research uses an RNA bearing a self-cleaving ribozyme sequence in ChIP assay. Cleavage of the nascent transcript leads to the observation that THO recruitment is RNA dependent, yet this dependency is not the cause for 5’ to 3’ increase in occupancy (Meinel et al., 2013). On the other hand, recent studies providing transcriptome maps of different mRNP biogenesis factors in *S. cerevisiae*, established THO binding across the whole mRNA length, with 5’ end enrichment and slight preference for longer transcripts (Tuck and Tollervey, 2013; Baejen et al., 2014). These results seem contradictory and further studies are necessary to link them into a joint recruitment/binding and function model for THO complex.

1.1.3.1.2 THO function

Members of the THO complex were first recognized for the observed transcription elongation impairment in null mutant strains, with the transcription-induced hyper-recombination as a specific characteristic (Aguilera and Klein, 1990; Piruat and Aguilera, 1998; Chavez et al., 2000). This latter phenomenon is caused by RNA:DNA hybrid formation (R-loops) behind the elongating Pol II, which leads to defective transcription elongation and blocks replication genome-wide, thus indicating that one of THO functional roles is to keep the nascent mRNA and the transcribed DNA apart (Huertas and Aguilera, 2003; Luna et al., 2005; Gomez-Gonzalez et al., 2011). Although genome-wide analyses demonstrate THO complex recruitment to all Pol II transcribed genes, the biggest impairment is exhibited in transcription of long, G + C rich, highly expressed genes and genes with internal repeats (Chávez et al., 2001; Voynov et al., 2006; Gomez-Gonzalez et al., 2011; Luna et al., 2012). Likewise, 5’ to 3’ increasing occupancy of THO was shown to have physiological importance

in expression of long transcripts (Meinel et al., 2013). This impairment is believed to be a consequence of reduced efficiency of transcription elongation (Rondon et al., 2003). Nonetheless, for the newest confirmed member of the THO complex, Tex1, no similar level of decreased gene expression or genome instability can be observed in a null mutant strain (Luna et al., 2005; Pena et al., 2012). Consistently, this mutant does not show the same importance for THO complex assembly nor its binding to nucleic acids (Pena et al., 2012).

Further insight into THO complex connection to proper assembly of the mRNP particle was obtained in Aguilera laboratory. An *hpr1-101* point mutant, which does not compromise the THO complex stability or chromatin recruitment but hinders recruitment of TREX subunit Sub2, was found to confer transcription impairment at a null mutant level, without triggering hyper-recombination (Huertas et al., 2006). In another study, the same group has shown that in this point-mutant transcription impairment is independent of R-loops formation, thus adding more importance to THO complex relevance in transcription and mRNP biogenesis (Gómez-González and Aguilera, 2009).

1.1.3.2 TREX complex

THO complex functions in co-transcriptional loading of mRNA export proteins Sub2 and Yra1, together forming the TREX complex and playing a key role in mRNA transport from the nucleus to the cytoplasm (reviewed in Oeffinger and Zenklusen, 2012). These proteins interact with THO complex physically and genetically, and are likewise conserved (Strasser et al., 2002). Their interconnection is further strengthened by the fact that THO and Sub2/Yra1 mutants show similar effect on transcription and hyper-recombination as well as a defect in poly(A) mRNA export (Jimeno et al., 2002). However, deletions of Tho2, Hpr1 and Sub2 lead to the strongest impairment in growth, transcription and export, revealing a hierarchy among TREX members (Garcia-Rubio et al., 2008).

1.1.3.2.1 Sub2

Sub2, a DEAD box RNA-dependent helicase, is a conserved functional splicing and export factor (Fan et al., 2001; Jensen et al., 2001a; Libri et al., 2001; Sträßer and Hurt, 2001). Sub2p is involved in multiple stages of mRNA maturation and its inactivation leads to nonproductive spliceosome assembly, decreased polyadenylation efficiency and mRNA

instability, as well as nuclear accumulation of poly(A) RNA (Saguez et al., 2013). Sub2 acts in the process of mRNP export in interaction with Yra1. Sub2 was at first believed to be the recruitment mediator for Yra1 (Sträßer and Hurt, 2001). However, alternative recruitment pathways for Yra1 have been discovered and the involvement of Sub2 has been determined in taking on Yra1 from the Pcf11, thus allowing the normal CFIA complex assembly and 3'end processing/termination (see next section 1.1.3.2.2). Sub2 is also involved in chromatin maintenance by the fact that its overexpression suppresses the DNA instability in THO mutant strains, possibly due to its helicase activity acting in the unwinding of RNA:DNA hybrid structures (R-loops) formed during transcription in these strains (Chavez et al., 2000; Gomez-Gonzalez et al., 2011; Saguez et al., 2013).

As already stated, Sub2 is recruited to the transcribed genes through interaction with the THO complex. This is supported by direct *in vitro* interaction with Hpr1, along with reduced Sub2 recruitment in *hpr1-101* and other THO mutant strains, as revealed by ChIP assay (Zenklusen et al., 2002; Huertas et al., 2006). Nevertheless, residual Sub2 recruitment and the fact that Sub2 overexpression suppresses phenotype defects of THO null mutant strains, argue the existence of an alternative, yet still undisclosed recruitment pathway (Fan et al., 2001; Zenklusen et al., 2002; Yu et al., 2012). Unlike THO complex, Sub2 recruitment was unambiguously shown to be RNA-dependent, which was proposed to reflect the 5' to 3' increasing ChIP profile of Sub2 (Abruzzi et al., 2004; Meinel et al., 2013).

Sub2 mutants convey most THO mutant phenotypes such as transcription and recombination defects, nuclear retention/degradation of aberrant transcripts and the formation of heavy-chromatin (Libri et al., 2002; Rougemaille et al., 2008; see page 27). Interestingly, both SUB2 deletion and overexpression under a strong promoter in wild-type (wt) background lead to mRNA export impairment (Sträßer and Hurt, 2001). This observation emphasizes the importance of THO complex in orchestrating the recruitment of protein factors and in transcript assembly into an export competent mRNP.

1.1.3.2.2 Yra1

Besides TREX connection to splicing through Sub2 function, this complex seems to be implicated in 3'end processing events by the action of Yra1, a member of evolutionary conserved family of RNA and export factor (REF) binding proteins (Zenklusen et al., 2002;

Johnson et al., 2009). Yra1 co-purifies as a part of the TREX complex, but the only direct contact was revealed with Sub2, which was believed to be the Yra1 transcription site recruitment mediator (Sträßer and Hurt, 2001; Zenklusen et al., 2002). However, this interaction was recently disproved as a cause for Yra1 recruitment, as Yra1 binding and recruitment reliance on the CFIA factor Pcf11 was discovered (Johnson et al., 2009). Indeed, the relevance of this interaction was reinforced by its implication in CFIA complex assembly, through competition for binding Pcf11 between Yra1 and CFIA member Clp1, with plausible influence on poly(A) site choice (Johnson et al., 2011; Haddad et al., 2012) (Figure 1.10). This model suggests Yra1 recruitment to transcription site through interaction with Pcf11, followed by Yra1 displacement by Clp1, facilitated through Yra1-Sub2 interactions and transfer onto mRNA (Johnson et al., 2009, 2011). Since complete CFIA complex is necessary for poly(A) site cleavage and mRNA release, this model can account for the aforementioned transcript cleavage/release defect when Sub2 is absent and not able to take on Yra1, thus preventing Clp1-Pcf11 binding and CFIA formation (Katahira, 2012; Oeffinger and Zenklusen, 2012). Another recent research demonstrated that Yra1 owns a CID domain through which it directly binds to Ser2/Ser5 diphosphorylated CTD *in vitro*. However, Yra1 CID also contains the nuclear localization signal (NLS), which makes it difficult to assess the importance of this interaction for Yra1 recruitment *in vivo* by simply deleting the CID domain (MacKellar and Greenleaf, 2011; Meinel et al., 2013).

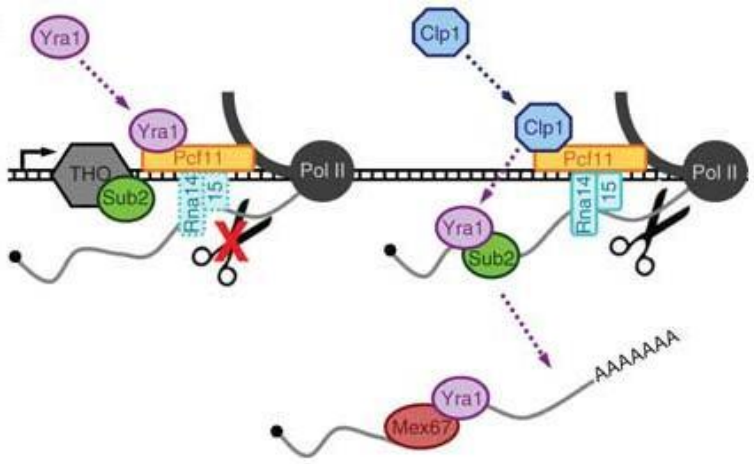


Figure 1.10: *Suggested model for Yra1 influence on co-transcriptional 3' end processing* (Johnson et al., 2011). During transcription, Yra1 is recruited to transcription site through interaction with Pcf11. At the 3' end of genes, Yra1 is transferred to Sub2 and thus clears the Pcf11 binding site for Clp1. Assembly of the complete CFIA complex enables poly(A) site cleavage (scissors) and mRNA release.

Recently, Yra1 was shown to interact with another DEAD-box helicase, besides Sub2. Genetic and physical interactions were observed between Yra1 and Dbp2, with Yra1 inhibition effect on Dbp2 helicase activity *in vitro*. Furthermore, Dbp2 was shown to function in *in vivo* mRNP assembly, by enabling loading of Yra1, Nab2 and Mex67 on the poly(A) transcript. A model was proposed where Dbp2 action is necessary to unwind the nascent transcript for proper mRNP assembly, after which Yra1 binding prevents further rearrangements by this helicase (Ma et al., 2013).

Concurrent with the finding of Yra1 binding to Sub2, Yra1 was also shown to bind Mex67, thus making it a RNA-binding adaptor of Mex67-Mtr2 heterodimer mRNA export receptor (Sträßer and Hurt, 2001). Since Sub2 and Mex67 interact with the same domain within Yra1, it is believed to be handed over from Sub2 to Mex67 in the process of forming an export competent mRNP (Bonnet and Palancade, 2014) (Figure 1.10). Mex67 is recruited to the transcription site through interaction of its C-terminal ubiquitin-associated (UBA) domain with ubiquitylated Hpr1 subunit of THO complex, followed by a transfer to the transcript along with its RNA-binding adaptors Yra1, Npl3 and Nab2, (Gwizdek et al., 2006; Hobeika et al., 2009; Babour et al., 2012). The final contribution of Yra1 to the mRNA in

export is its dissociation from the mRNP prior to NPC passage, which is promoted by Yra1 ubiquitination and presents a possible crucial step in establishing an export-competent mRNP (Iglesias and Stutz, 2008; Iglesias et al., 2010).

1.1.3.2.3 TREX function

In addition to implication of THO complex in transcription elongation (see section 1.1.3.1.2), another class of transcripts was discovered in THO/Sub2 mutant strains, which are truncated at the 3'end and/or retained at the transcription site (Jensen et al., 2001a; Libri et al., 2002; Vinciguerra and Stutz, 2004) (Figure 1.11). Most of the research of this model was performed by observing heat shock mRNAs after a growth shift to 37˚C, namely the *HSP104* transcript. Fluorescent *in situ* hybridization (FISH) assay enables the visualization of *HSP104* retention dots in THO/Sub2 mutants. In Libri et al., 2002 the retention dots were detected even with the use of FISH probes targeting the *HSP104* sequence just upstream of the stop codon. This suggests that the retained transcripts are complete or nearly complete. In the same strains and experimental conditions, the nuclear accumulation of poly(A) RNA can be detected. However, the same study determined another subset of *HSP104* transcripts in THO/Sub2 mutants by Northern blot of total RNA from cells grown at 37˚C. These transcripts are truncated at the 3'end, which was determined to be the result of a 3' to 5' degradation by the Rrp6 exonuclease. Indeed, deletion of the RRP6 gene from the THO/Sub2 mutant strains leads to restoration of HSP104 3'end levels, but also results in disappearance of the HSP104 retention dots in the same double mutant strain. This is believed to be a result of transcription quality control step, which will be presented in more details in section 1.2.3.

The two proposed models explaining the origin of 3' end truncated transcripts in TREX mutant strains are, however, not mutually exclusive. In the view of the literature regarding each of the two models, we can observe a major difference in experimental methods which could account for the discrepancy of the obtained results. Experiments supporting the transcriptional model (Figure 1.11, section 1.1.3.1.2) were carried out at optimal temperature for yeast growth. In this condition the function of THO in keeping the nascent transcript and the DNA apart is accentuated, as well as its importance in efficient transcription elongation (Jimeno et al., 2002; Rondon et al., 2003). However, the exosomal model, described in previous paragraph, focuses on the effect of THO mutations on a transcript induced in heat-shock conditions. In these conditions the transcription rate is upregulated, while RNAs exhibit

strong nuclear retention. A possible result of heat shock could be the polymerase overload on the transcribed gene (described in the next paragraph) which would diminish the necessity of THO complex in preventing RNA:DNA hybrid formation and stress its function in proper mRNP biogenesis. Consequently, production of defective mRNPs would activate nuclear surveillance machinery which would retain and degrade the affected transcripts. The fact that both phenotypes observed in THO mutant strains, hyper-recombination and/or transcript retention and degradation, can partially be alleviated by slowing down the rate of transcription, suggests THO function in securing optimal kinetics for mRNP assembly and contribution to transcription speed and efficiency (Jensen et al., 2004; Jimeno et al., 2008).

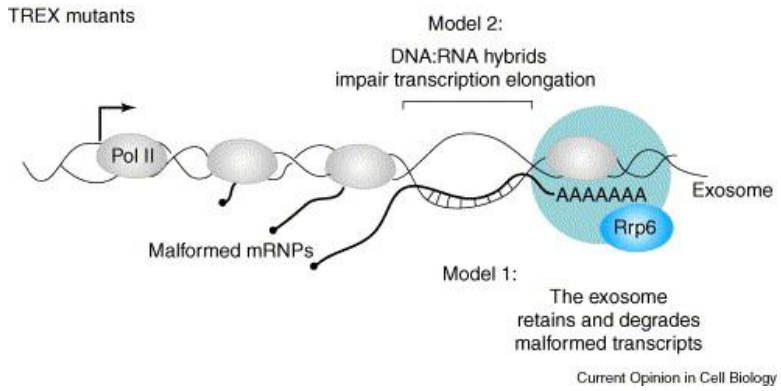


Figure 1.11: *Two proposed models explaining the origin of 3' end truncated transcripts in TREX mutant strains* (Vinciguerra and Stutz, 2004). The exosomal model (model 1) suggests that mutations in TREX lead to aberrant mRNP formation from fully synthesized transcripts, which are then retained at transcription site and degraded by the nuclear exosome. The transcriptional model (model 2) states that the lack of TREX complex leads to formation of DNA:RNA hybrids behind the elongation Pol II which impairs transcription and causes DNA hyper-recombination and genome instability. However, both models agree on the importance of TREX in mRNP formation and are not necessarily mutually exclusive.

Additional importance of THO complex in promoting high pace transcription was determined by Libri group in collaboration with Jensen and Stutz groups. They found that under heat shock conditions THO null mutants exhibit accumulation of large protein-nucleic acid aggregates, referred to as the heavy chromatin. This formation contains stalled mRNP intermediates, along with nuclear pore components and polyadenylation factors associated with chromatin. During the chromatin extraction step in ChIP assay, the target heat shock sequences were absent from the THO mutant strains preparations. They were found to be sequestered in the pellet fraction during high speed centrifugation step, as a part of heavy chromatin complex. Hence, this phenomenon was termed differential chromatin fractionation (DCF). Heavy chromatin formation was found at the 3'end of ~400 genes, depending on the presence of a functional terminator. CPF and CFIA mutants defective for polyadenylation and transcription termination, respectively, abolished heavy chromatin formation. However, the most important determinant of heavy chromatin formation is the nature of the gene promoter. The high-power promoter firing leads to polymerase overload on the transcribed genes which are docked to the NPC. Consequently, this overwhelms the 3'end processing and transcript

release and leads to DCF (Figure 1.12). Taken together, these results affirm THO complex role in coordinating rate of transcription with the downstream processes, 3'end processing/termination and transcript release from the transcription site (Rougemaille et al., 2008; Mouaikel et al., 2013)

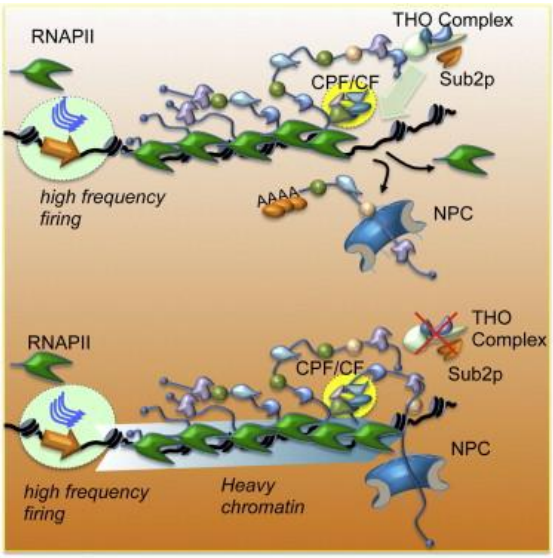


Figure 1.12: *Model of heavy chromatin formation in THO-Sub2 mutants* (Mouaikel et al., 2013). In wt strains THO-Sub2 complex coordinates different processing events and promotes optimal kinetics of transcription. In the absence of THO-Sub2, transcription kinetics is disturbed which leads to an overflow with transcribing polymerases reaching the end of a gene, piling up and docking to the NPC.

In accordance with these findings, mutation of THO complex was shown to impede 3'end processing factors' release from the mRNP and lead to inefficient polyadenylation (Saguez et al., 2008; Qu et al., 2009). In addition to direct contribution of THO complex to transcription, mRNP assembly and possibly transcript processing events (reviewed in Luna et al., 2012), this complex also recruits and interacts with other protein factors, notably the ones essential for mRNA export, thus playing a major role in production of mature, export-competent mRNPs.

1.1.3.2.4 Other THO/TREX interactions

Two serine-arginine (SR) rich, poly(A) RNA-binding proteins, Gbp2 and Hrb1, co-purify with other members of the TREX complex, but they are not essential for the assembly

of this complex (Hurt et al., 2004). This interaction accounts for their co-transcriptional recruitment to active genes. Gbp2 and Hrb1 bind along the whole transcript length, with 5'end enrichment, as do other THO/TREX members, however, they do not show the same 5' to 3'end increasing chromatin recruitment pattern (Reed and Cheng, 2005; Meinel et al., 2013; Tuck and Tollervey, 2013). To date, no research was published which would show whether Gbp2 or Hrb1 mutants share the phenotype indicated for other THO/TREX members. Recently, these two factors were shown to preferentially bind transcripts derived from intron-containing genes. They serve as splicing surveillance factors and stay bound to the export-competent transcript during its passage through the NPC and into the cytoplasm (Hackmann et al., 2014).

TREX maintains a functional interaction with another protein complex involved in coupling transcription elongation to mRNA export. This complex comprises Thp1-Sac3-Sus1-Cdc31, with Sem1 as the newest characterized member, together forming THSC, also named TREX-2 complex (Köhler and Hurt, 2007; Gonzalez-Aguilera et al., 2008; Faza et al., 2009). Similar to THO, transcription defect, genetic instability coupled with R-loops formation and impaired 3'end processing can be observed in TREX-2 mutants (Gonzalez-Aguilera et al., 2008; Rondón et al., 2010). However, only TREX-2 interacts with the NPC, and Sub2 overexpression is lethal in TREX-2 mutants (Rondón et al., 2010). It is proposed that the two complexes function at different steps of the same pathway, coupling transcription and export, and providing a feedback mechanism for control of transcription and genetic integrity (Gonzalez-Aguilera et al., 2008; Luna et al., 2012) (Figure 1.13).

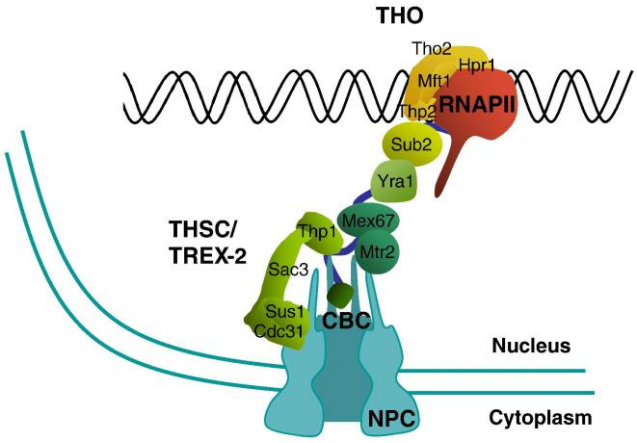


Figure 1.13: *THO/TREX and TREX-2 function in the same pathway from transcription to export of mature mRNPs* (Rondón et al., 2010).

1.2 mRNP decay and nuclear quality control in yeast

Opposite from the enduring, information bearing DNA whose lifespan is paralleled with the life of the whole cell, RNA is a molecule with versatile functions and a regulated turnover. mRNA life-cycle starts with previously described steps of transcription, processing and export, followed by translation and decay in the cytoplasm. mRNA turnover defines cell growth, differentiation and environmental response (Ross, 2001; Haimovich et al., 2013). In addition to decay at the end of an RNA life-cycle, it can be degraded at earlier steps if it is found to be aberrant and not able to execute its function (reviewed in Parker, 2012).

1.2.1 mRNA degradation

After serving their purpose as information carriers for protein synthesis in the cytoplasm, mRNAs are degraded in a turnover process in that same cellular compartment. Possible degradation pathways in the turnover process are defined as general default decay pathways (Das and Das, 2013). These are initiated by mRNA deadenylation, leaving an unprotected oligo(A) 3'end (Norbury, 2013). Most transcripts are subsequently decapped and then degraded by Xrn1 nuclease in the 5' to 3' exonucleolytic pathway, while others are subjected to 3' to 5' exonucleolytic degradation pathway by cytoplasmic exosome (Das and Das, 2013).

Another kind of degradation pathways who function selectively to terminate transcripts recognized as aberrant are termed specialized mRNA decay pathways and they take place in the cell nucleus, as well as in the cytoplasm (Houseley and Tollervey, 2009) (Figure 1.14). Aberrant mRNPs are recognized during quality control (QC) surveillance of mRNP biogenesis. A QC check-point at each processing step and at the NPC in the nucleus of eukaryotes makes sure the mRNP is export-competent (Tutucci and Stutz, 2011; Eberle and Visa, 2014). Degradation of aberrant transcripts in the nucleus can be carried out by two pathways: the minor - 5' to 3' degradation by Rat1 exonuclease after decapping of the transcript; and the major pathway – carried out by the 3' to 5' exonucleolytic action of the nuclear exosome (Tutucci and Stutz, 2011). The nuclear subset of QC processes will be presented in further details in section 1.2.3.

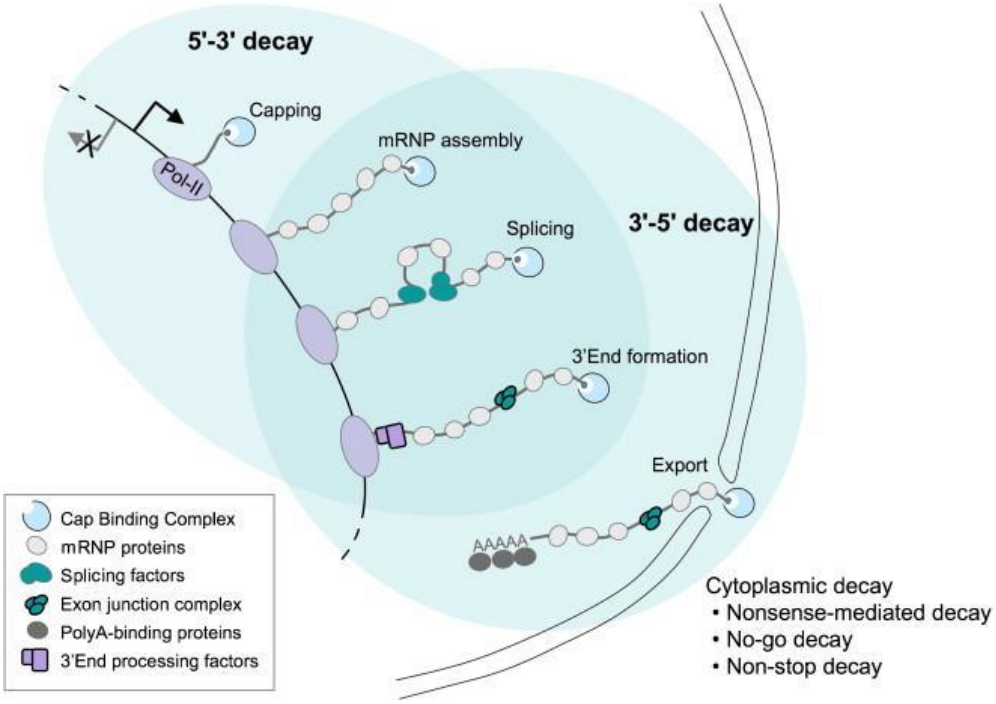


Figure 1.14: *Decay systems in different nuclear and cytoplasmic QC mechanisms* (Eberle and Visa, 2014). Most 3' to 5' exonucleolytic and endonucleolytic degradation in the nucleus is carried out by the nuclear exosome, while 5' to 3' decay is performed by Rat1 and DOX exonucleases. Their cytoplasmic counterparts are cytoplasmic exosome for 3' to 5' and Xrn1 for 5' to 3' decay.

At the cytoplasmic side quality control mechanisms act in response to difficulties encountered in the process of translation (Doma and Parker, 2007). Adaptor proteins interact with translation machinery and direct aberrant mRNPs into different degradation pathways: Nonsense-mediated, No-go and Non-stop decay pathways, which have been extensively reviewed by Parker, 2012. Nonsense-mediated decay acts in response to faulty translation termination, caused by a variety of events such as long 3' untranslated region (UTR), alternative translation initiation sites, upstream open reading frames (ORFs), presence of introns with stop codons and translation frameshift. In this pathway, aberrant transcripts are decapped or deadenylated and degraded in 5' to 3' or 3' to 5' end manner, respectively, and coupled to repression of translation. In the No-go decay pathway, stalled translation leads to endonucleolytic transcript cleavage by a still unknown endonuclease. The remaining mRNA fragments are degraded in both 5' to 3' and 3' to 5' directions, by Xrn1 and exosome, respectively. Finally, if for different reasons transcript does not contain a stop codon, 3' end

ribosome stalling triggers its rapid degradation from 3’ to 5’ by the exosome in the Non-stop decay pathway (Parker, 2012).

1.2.2 The exosome

The eukaryotic RNA exosome is a multisubunit complex with a highly conserved core structure. Indeed, the core structure homologues reach to *Archaea* and *Eubacteria* (Lykke-Andersen et al., 2011). 9 subunits of a barrel-like core form a two-layered ring: the bottom hexamer (Rrp41, Rrp42, Rrp43, Rrp45, Rrp46 and Mtr3), and the upper RNA binding cap (Rrp4, Rrp40 and Csl4) (Chlebowski et al., 2013; Schneider and Tollervey, 2013) (Figure 1.15). However, in yeast and humans the exosome core itself is catalytically inactive. Still the core is indispensable for the nuclease activity carried out by two associated components, Dis3/Rrp44 and Rrp6 (Wasmuth and Lima, 2012) (Figure 1.15). In budding yeast, Dis3 subunit accompanies the core in both the nucleus and the cytoplasm, while Rrp6 is confined to provide catalytic activity only in the nucleus. Hence, the exosome exists in two isoforms: cytoplasmic (core + Dis3) and nuclear (core + Dis3 +Rrp6) (Chlebowski et al., 2013).

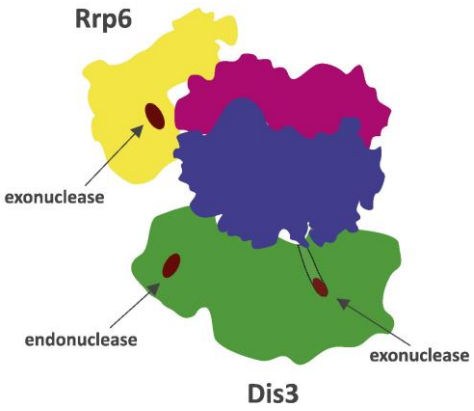


Figure 1.15: *Schematic representation of the eukaryotic nuclear exosome complex* (Chlebowski et al., 2013). The barrel-like core is depicted in blue (hexamer) and magenta (cap). The two catalytical components are represented in yellow (Rrp6) and green (Dis3) with the active sites denoted in red.

Dis3 possesses two nuclease activities: endonuclease, which may act on substrates arriving through the central channel of the exosome core or directly from the surroundings, and the 3' to 5' exonuclease activity, whose active site is placed at the very bottom of the

central channel (Makino et al., 2013) (Figure 1.15). Although Rrp6 is the only non-essential subunit of the exosome, its presence in the exosome complex enhances Dis3 activities, and the two nucleases seem to have overlapping, as well as distinct degradation targets (Gudipati et al., 2012; Schneider et al., 2012; Wasmuth and Lima, 2012). Rrp6 is a 3' to 5' exonuclease, placed on the exosome cap, at the top of the central channel (Figure 1.15). Its function is dependent on the core to some extent, since substrate RNA seems to enter the channel and then go through the openings between the cap and the hexamer to reach the Rrp6 active site (Wasmuth et al., 2014). Nevertheless, Rrp6 was shown to carry out some exonucleolytic functions *in vivo* even whilst physically uncoupled from the exosome (Callahan and Butler, 2008). Altogether, Rrp6 was shown to function in pre-rRNA processing, 3' maturation of small stable RNAs, degradation of CUTs and mRNA QC (Butler and Mitchell, 2010).

Previously mentioned roles of the exosome in transcript degradation present only a small part of its vast functions *in vivo*. In the cell nucleus, the exosome acts on different RNA substrates providing their processing, maturation or degradation. Recognition and specific processing of these substrates is facilitated by different cofactors/activators interacting with the exosome (Schneider and Tollervey, 2013).

1.2.2.1 Exosome associated factors

Mtr4 is an essential nuclear co-factor of the yeast exosome. It is a helicase with the role of 3' to 5' unwinding the RNA substrate for degradation in direct interaction with the exosome, or as a part of the Trf4/5-Air1/2-Mtr4 polyadenylation (TRAMP) complex (see section 1.2.2.2) (Bernstein et al., 2008). Mtr4 was found to partake in exosome function with or without dependency on the Rrp6 subunit. Recent research indicate Rrp6, together with another exosome co-factor Rrp47, takes part in providing a binding site for Mtr4, although other interaction mechanisms between Mtr4 and exosome cannot be excluded (Klauer and Hoof, 2013; Schuch et al., 2014).

Rrp47/Lrp1 is a nuclear exosome-associated RNA binding protein, shown to form a heterodimeric complex with Rrp6, which leads to structural rearrangements in both proteins and modulates their activities (Garland et al., 2013; Dedic et al., 2014; Schuch et al., 2014). Indeed, deletion mutants for either of the two proteins leads to the observation of similar defects, further supported by discovered interdependence for *in vivo* stabilization and/or

expression between the two factors (Feigenbutz et al., 2013a, 2013b; Stuparevic et al., 2013). As mentioned above, Rrp6-Rrp47 complex provides a binding surface for Mtr4, while a third co-factor, Mpp6, binds the exosome independently (Schuch et al., 2014) (Figure 1.16).

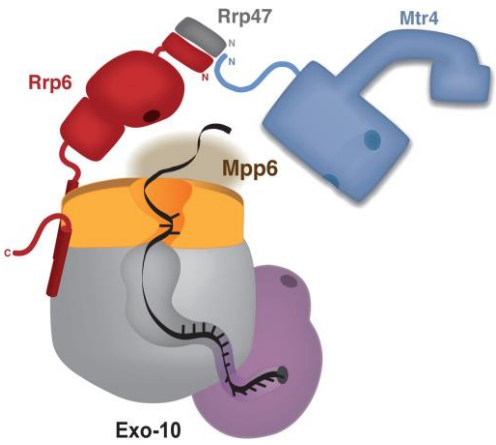


Figure 1.16: *Model of the nuclear exosome complex and its associated factors* (Schuch et al., 2014). In orange and gray are depicted the cap and hexamer part of the core exosome, respectively. Dis3 is at the bottom part of the hexamer shown in purple with active sites highlighted as dark circles. Rrp6 is shown in red with its C-terminal bound to the exosome and the N-terminal interacting with Rrp47 (gray), thus providing a binding platform for Mtr4 (blue). Provisionary placement of Mpp6 is at the top of the core exosome.

Mpp6 is also an RNA binding protein associated with the nuclear exosome complex which was found to have synthetic lethal interactions with both Rrp6 and Rrp47 (Milligan et al., 2008). This factor was shown to preferentially bind pyrimidine-rich RNA and to function in rRNA maturation, degradation of CUTs and mRNA surveillance. Nonetheless, its exact role in exosome function is poorly understood (Butler and Mitchell, 2010; Stuparevic et al., 2013).

1.2.2.2 Exosome associated complexes

As stated above, exosome co-factor Mtr4 also functions as a part of the exosome activating complex TRAMP, in conjunction with one poly(A) polymerase, Trf4 or Trf5, and an RNA binding protein, Air1 or Air2 (LaCava et al., 2005; Wyers et al., 2005). Accordingly,

in vivo TRAMP exists in two forms with only partially redundant functions: Trf4-Air2-Mtr4 (TRAMP4) and Trf5-Air1-Mtr4 (TRAMP5), with TRAMP4 being the pre-dominant form (Porrua and Libri, 2013b). Together with the exosome, TRAMP functions in transcript degradation and surveillance, as well as in RNA 3'end formation (Schmidt and Butler, 2013). The key activities of this complex seem to be the addition of a short (4-5 adenosines) poly(A) tail to the substrate RNA, coupled to helicase activity of Mtr4, that needs a minimal 3' overhang to bind its substrate (Schneider and Tollervey, 2013). Indeed, recent *in vitro* studies have revealed a possible reciprocal coordination of TRAMP subunits Trf4 and Mtr4: (a) Trf4 polyadenylation presumably serves to prime the transcript for Mtr4; (b) Mtr4 seems to modulate Trf4 activity which in turn prevents the formation of a poly(A) longer than necessary for priming; and (c) Trf4 directly stimulates Mtr4 helicase activity (Jia et al., 2011, 2012; Taylor et al., 2014). These studies suggest a substrate first interacts with Trf4 which primes it and transfers it to Mtr4. This has recently been confronted by the research of TRAMP structure from which it is evident that the substrate passage from Mtr4 to Trf4 is more probable (Falk et al., 2014). Additional research on both Trf4-Mtr4 interplay and TRAMP structure are necessary.

Termination complex Nrd1C (previously described in section 1.1.2.2) is another exosome assisting complex. Nrd1 couples transcription termination to transcript processing and degradation by recruiting the TRAMP-exosome complex to sn/snoRNA and CUTs (Vasiljeva and Buratowski, 2006; Schmid and Jensen, 2013). Air2 was shown to co-purify with Nrd1-Nab3 (Schmidt and Butler, 2013). However, the mechanism of this interaction is still unknown. Recently, a mechanism of interaction between Nrd1C and Trf4 was defined. Trf4 seems to possess a CTD mimicking Nrd1 interacting motif (NIM), which binds to Nrd1-CID, as does the Pol II CTD, but in a mutually exclusive manner (Tudek et al., 2014). The same study proposes that this alternative binding of Nrd1C is the base for double mechanism of Nrd1C function in transcription termination and transcript degradation. Newly discovered Nrd1 binding specificity for wide range of RNAs suggests a possible function of Nrd1 as a general RNA-binding subunit of TRAMP-exosome in its processing/degradation performance (Bacikova et al., 2014). The interaction between these two complexes has been most extensively studied in transcription quality control, which will be presented below.

The newest discovered complex interacting with the nuclear exosome complex is the Ccr4-Not complex. This complex comprises at least nine subunits and is implicated in a vast

number of different cellular processes, such as transcription, RNA processing, export, translation and protein degradation (reviewed in Collart and Panasenko, 2012). This complex was shown to interact physically and genetically with TRAMP-exosome complex (Azzouz et al., 2009) and its possible involvement in mRNA QC has been suggested (Assenholt et al., 2011; Miller and Reese, 2012).

1.2.3 Nuclear quality control

The mRNP biogenesis described in preceding sections of this introduction is a mere glimpse into all the data obtained to date on this subject, and probably not even the tip of the iceberg which is the functioning of mRNP biogenesis *in vivo*. A high complexity of this process reflects its biological significance, which is further emphasized by the concomitant presence of quality control steps on its path (Figure 1.17).

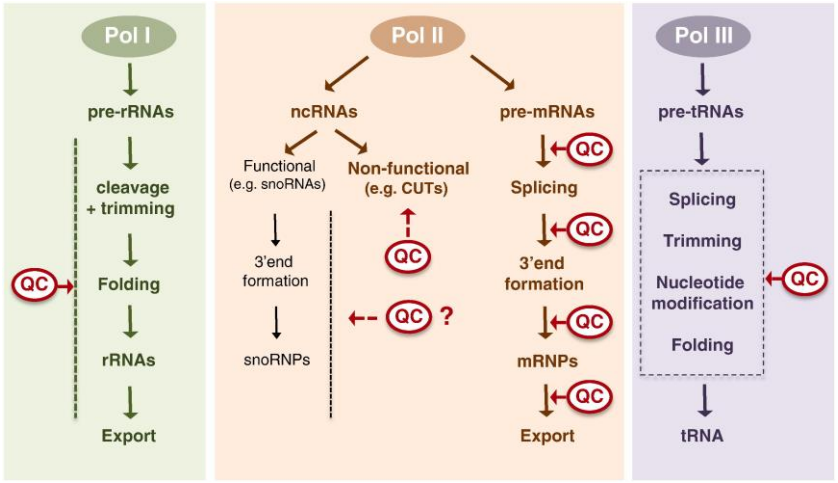


Figure 1.17: *Quality control mechanisms in transcription pathways of the three RNA polymerases* (Porrua and Libri, 2013b).

1.2.3.1 QC of mRNA processing

If the emerging nascent transcript fails to be properly capped, it cannot bind CBC complex providing protection from enzymatic attack. In *S. cerevisiae* such transcript is decapped by the action of several enzymes, Dcp1/2, Dox1 and Rai1, which catalyze removal of different cap intermediates. The end product of decapping is a transcript with a monophosphorylated 5'end which is targeted for 5' to 3' exonucleolytic degradation by Rat1 (Jurado et al., 2014).

Splicing and 3' end formation defects in yeast lead to transcript retention and degradation by the nuclear exosome (Eberle and Visa, 2014). Unspliced transcripts are believed to be retained in the nucleus by some NPC proteins, although the recognition mechanism was unknown until recently (Tutucci and Stutz, 2011). Newly identified quality control factors for spliced mRNA are THO/TREX interacting proteins Gbp2 and Hrb1, shown to retain mRNA until completion of splicing and recruitment of export factor Mex67, or until its degradation following TRAMP-exosome recruitment (Hackmann et al., 2014).

Mutations of 3' end processing and polyadenylation factors lead to defective transcription termination and polyadenylation, respectively, and are targeted for degradation by the nuclear exosome (Libri et al., 2002; Milligan et al., 2005). As pointed out earlier (section 1.1.3.1.2) 3'end processing events are tightly coupled to export-competent mRNP formation, and notably to THO/TREX function.

1.2.3.2 QC of mRNP assembly

The nuclear quality control of transcripts seems to be governed by the nuclear exosome subunit and quality control factor, Rrp6. Indeed, this factor is necessary for establishing all of QC phenotypes – transcript degradation, nuclear retention and DCF formation – observed in the most commonly used model for study of quality control – HSP104 transcript in THO mutant strains (Libri et al., 2002; Rougemaille et al., 2007; Assenholt et al., 2008; Rougemaille et al., 2008). Experiments in this model show that aberrant mRNPs are dealt with in two ways: one subpopulation is quickly degraded, while the other one is retained in nuclear foci at transcription site and slowly leaks into the cytoplasm (Libri et al., 2002; Rougemaille et al., 2007; Kallehauge et al., 2012). The same nuclear

retention phenotype has been observed for other mutants in the mRNP assembly/export pathway (Sub2, Yra1, Mex67 and others) and also for CFIA factors (Jensen et al., 2001b; Libri et al., 2002; Zenklusen et al., 2002; Rougemaille et al., 2007).

Production of aberrant transcripts in THO/TREX mutants is "toxic" in yeast, as observed by the reduced growth, and this phenotype is more pronounced at higher temperatures (Garcia-Rubio et al., 2008; Libri et al., 2002). In combination with deletion of Rrp6, which leads to transcript release from nuclear foci, this phenotype is further exacerbated. At high temperatures (above 30˚C) the accumulation of aberrant transcripts seems to "suffocate" the cells, making them inviable (Libri et al., 2002). A novel study presents a functional role of nuclear site retention and slow cytoplasmic release in the state of decreased mRNP maturation capability. It suggests that this retention serves in providing additional time for processing necessary for transcripts to reach translational competence (Kallehauge et al., 2012). Indeed, Kallehauge et al., (2012) show that transcripts retained in nuclear foci in export dysfunctional *mex67-5* mutant strain are slowly released, exported and fitted for translation. However, forced export by Sub2 overexpression causes transcript dissipation from the foci and only brings upon an increased growth deficiency, elevated by concomitant Rrp6 overexpression and foci restoration.

Recently, in our laboratory a new and original experimental approach to study nuclear mRNP surveillance was implemented (presented in section 1.3). Results obtained with this new assay provide further insight into the involvement of Rrp6 in mRNP QC. Nrd1 was shown to assist Rrp6 co-transcriptional recruitment and targeting of aberrant transcripts which leads to transcript retention and degradation (Honorine et al., 2011; unpublished results). This targeting and degradation are further facilitated by exosome co-factors Rrp47, Mpp6, as well as the TRAMP complex (Stuparevic et al., 2013). Intriguingly, TRAMP was shown to be involved in two distinct forms, bearing either Trf4 or Trf5, but both interacting with Air2 RNA-binding component, while Air1 was found to be dispensable. These complexes may contain Mtr4 but its helicase activity is not involved into the QC process.

1.2.3.3 Specific and/or competitive QC

Specific recognition of degradation substrates by the exosome has been characterized for certain transcripts, i.e. CUTs, while for others, such as unspliced mRNAs – easily

distinguishable from mature forms and where such specificity mechanism is not hard to envisage – the recognition process has not been determined yet. In the case of THO/TREX mutants, where the nature of the defect is not completely understood and a specific recognition system has not been demonstrated, a kinetic competition between processing and QC seems plausible. In this competition model, degradation and processing compete for substrate RNA, and do not take into account if the transcript is defective or not. (Porrua and Libri, 2013b)

In support of a competitive model, along with previously mentioned THO/TREX dependence on transcription rate (see section 1.1.3.1.2), a recent study showed that even in wt strains, a large subset of potentially functional RNA molecules is degraded by the nuclear exosome, probably prior to achieving full maturity, and this is especially pronounced for tRNA precursors (Gudipati et al., 2012). The RNAs degraded in non-mutant conditions, or "the Angels' share" of RNA, is believed to be of double origin: the first group are defective RNAs resulting from random errors, consequently slowing their processing rate and making them more prone to exosome degradation, while the second group are normal RNA molecules in the case when degradation outcompetes normal processing steps (Porrua and Libri, 2013b). Finally, it is argued that maintenance of non-optimized mRNP biogenesis, coupled with generic QC is more economical from the evolutionary and regulatory perspectives, than the costly development of highly efficient processing systems (Gudipati et al., 2012; Porrua and Libri, 2013b).

1.3 Bacterial factor Rho as a tool to study mRNP biogenesis and nuclear quality control in *Saccharomyces cerevisiae*

Transcription termination in bacteria is executed by two termination mechanisms, one of them being mediated by a conserved factor Rho, accounting for 20 – 50% of transcription termination events in *Escherichia coli* (D'Heygere et al., 2013). In addition to transcription termination, in recent years novel functional roles of Rho have been uncovered, such as regulation of gene expression, prevention of R-loop formation and maintenance of genome stability (reviewed in Boudvillain et al., 2013).

1.3.1 Structure and function of Rho factor

Rho factor is a ring-shaped homohexamer helicase, with each subunit made of 419 amino acids and with no known functional homolog in eukaryotes (Geiselmann et al., 1992). Rho binds RNA through its N-terminal domain, so-called primary binding site (PBS). The PBS recognizes and binds to *rut* (Rho utilization) sequence or generally any C-rich sequence exposed on the nascent RNA following ribosome release at the end of a coding sequence or upon the encounter of a non-sense codon (Yu et al., 2000). Loading of Rho onto the RNA follows, with positioning of the RNA into the central channel of the ring structure, shown to have enough space to accommodate a single RNA chain (Skordalakes and Berger, 2003) (Figure 1.18A). Rho factor exists in two forms, as a split-open and closed-ring with the closed form predominance in the presence of an RNA ligand (Gogol et al., 1991). In the central channel the RNA binds to the secondary binding site (SBS) of the Rho C-terminal domain which also bears the ATPase activity necessary for the Rho 5' to 3' translocation along the nascent RNA (Bear et al., 1985; Thomsen and Berger, 2009) (Figure 1.18B). Crystal structures of the Rho factor loaded on an RNA substrate reveal two distinct organizations of RNA:SBS contacts within the closed-ring form of the hexamer and suggest different models of RNA translocation (Skordalakes and Berger, 2006; Thomsen and Berger, 2009). However, most recent research support the "asymmetric" ring structure of Rho and the "escort" model of RNA translocation which suggests translocation in monotonous steps of one nucleotide per molecule of hydrolyzed ATP (Thomsen and Berger, 2009; Rabhi et al., 2011; Soares et al., 2014).

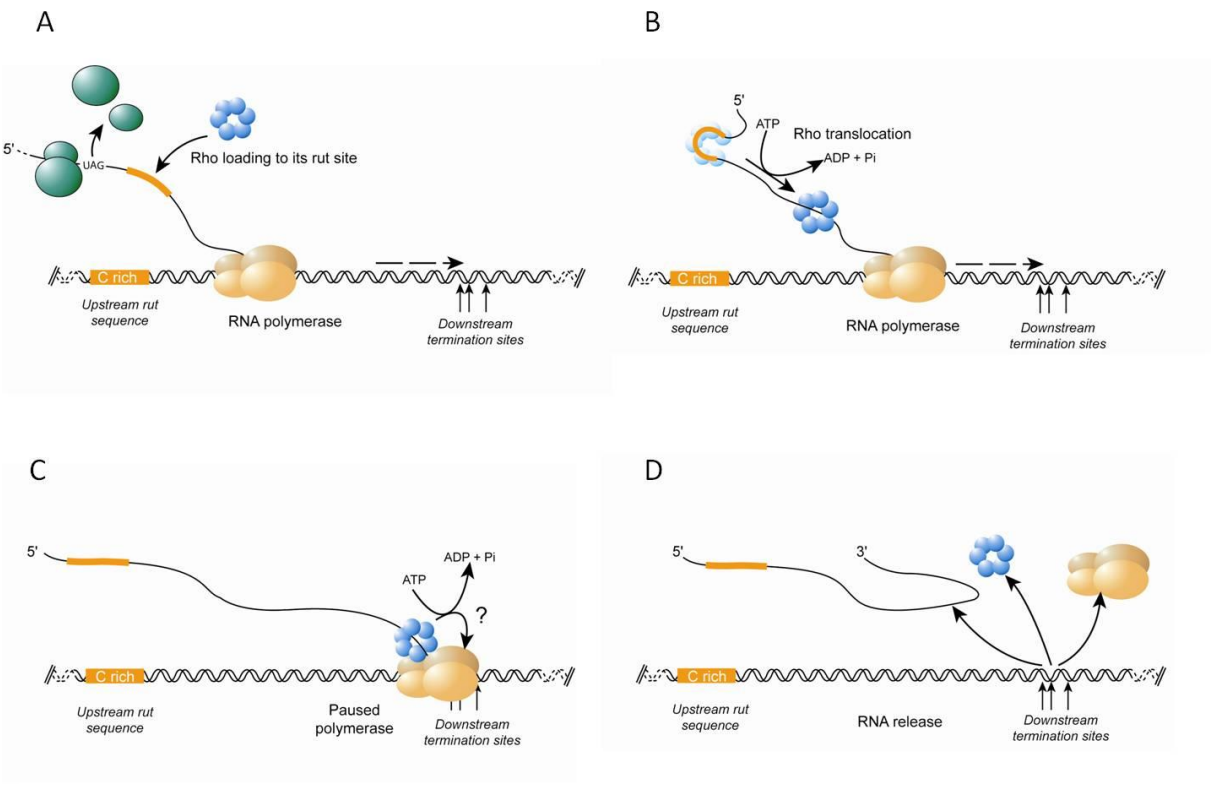


Figure 1.18: *Model of Rho-dependent transcritpion termination in E.coli.* (A) In the course of transcription, Rho recognizes and loads onto the exposed *rut* sequence or any other C-rich sequence on the nascent RNA. (B) ATP driven translocation of Rho towards the transcribing polymerase. By the tethered tracking model not depicted here, Rho tracks along the RNA continuously bound to the loading site. (C) According to the kinetic coupling model, the efficiency of transcription termination depends on the differences between Rho translocation rate and RNA polymerase transcription rate enabling the Rho factor to "catch up" with the polymerase which (D) promotes RNA polymerase dissociation from the DNA template and transcript release by a mechanism that is still highly debated.

The current transcription termination model suggests Rho loads onto the RNA at a *rut* site, binds the transcript at the SBS which promotes conformational changes to a closed form, catalytically competent for ATP hydrolysis. The RNA-dependent ATPase activity provides the energy for 5' to 3' translocation of Rho factor towards the transcribing polymerase, with the PBS still bound to the loading site (tethered tracking model) (Koslover et al., 2012; Gocheva et al., 2015). The efficiency of transcription termination highly depends on the rate differences in translocation and transcription of Rho factor and the Pol II, respectively. Indeed, this "kinetic coupling" model provides a powerful way to modulate termination by slight variations in the relative rates of the two enzymes (Jin et al., 1992; Gocheva et al.,

2015). Once the Rho factor reaches the polymerase paused at the transcription termination site, it promotes its dissociation from the DNA template and the release of transcript through a mechanism whose details still remain obscure (Ciampi, 2006) (Figure 1.18C and D). Three different mechanisms have been proposed for Rho induced termination. In the first, the RNA is believed to be "pulled" away by the Rho helicase activity until it is uncoupled from its DNA template (Richardson, 2002). The second mechanism suggests that Rho promotes the forward translocation of the polymerase, thus "pushing" it of the DNA template (Park and Roberts, 2006). The third, somewhat controversial model, suggests a Rho induced allosteric change of the elongation complex resulting in its dissociation and termination of transcription (Epshtein et al., 2010; Boudvillain et al., 2013).

Along with its primary function in transcription termination, Rho has been implicated in regulation of gene expression and maintenance of genome stability in prokaryotes (Boudvillain et al., 2013). General physiological role of Rho in terminating transcription of genes with reduced translational rates (Rho-dependent transcriptional polarity) has been expanded with a finding that *trans*-encoded sRNA can also induce this Rho polarity in its bicystronic target gene by inhibiting translation initiation and allowing Rho to gain access to *rut* binding sites in the mRNA (de Smit et al., 2008). Rho then causes a premature transcription termination which concomitantly reduces expression of the gene encoded by the second cistron. Another mechanism of modulating gene expression through involvement of Rho factor was discovered in couple with bacterial riboswitch control of gene expression (Hollands et al., 2012). In this mechanism the riboswitch includes a *rut* sequence which is available, or not, for Rho binding, depending of the secondary structure of the riboswitch. Thus, Rho factor induces transcription termination when riboswitch structure allows its binding to the *rut* site. Rho factor also plays a role in maintaining genome stability by suppressing pervasive antisense transcription, preventing the formation of R-loops and limiting RNA polymerase backtracking to avoid replication-transcription collisions. In this way Rho action diminishes possible replication stress, DNA breakage and hyper-recombination (Grylak-Mielnicka et al., 2016).

Rho was proven to act as a powerful molecular motor, capable of unwinding nucleic acid structures and dissociating RNA-protein complexes present in its path (Walmacq et al., 2004, 2006). Indeed, Rho has an ability to disrupt the biotin-streptavidin interaction if it is present on its path on an RNA substrate. Although this ability was proven *in vitro*, this

heterologous interaction is stronger than most nucleic acid-protein interactions *in vivo* (Schwartz et al., 2007a). Other *in vitro* and *in vivo* studies showed that for Rho loading onto RNA, any sequence with adequately spaced C residues or certain RNA structures can suffice (Guérin et al., 1998; Schwartz et al., 2007b). This features of Rho factor, as well as its ability to induce release of yeast Pol II paused at a transcription termination site *in vitro* (Lang et al., 1998), prompted its expression *in vivo* in yeast, where it was shown to serve as a useful tool to study QC of transcription.

1.3.2 An original experimental system to study nuclear QC

In our laboratory the interesting functional features of Rho factor led to the hypothesis that once expressed in yeast, Rho could preserve its features, track along the nascent RNA and impair normal mRNP biogenesis. The experimental system was set up by placing Rho encoding sequence, coupled with C-terminal NLS, under Dox-regulated $TetO_7$ promoter within a centromeric plasmid also harboring a constitutively expressed transactivator tTA.

Our initial study on expression of a bacterial transcription termination factor Rho in yeast cells, showed that Rho expression is toxic in *S. cerevisiae* and the observed growth defect was dependent on Dox concentration in the medium (Mosrin-Huaman et al., 2009). Moreover, expression of a Rho mutant in yeast, with a reduced ability to terminate transcription *in vitro* and *in vivo* (Rho-KE), showed that this function of factor Rho is crucial for inducing the growth defect phenotype upon its expression in yeast. On a molecular level, Rho presence lowered the steady-state levels of Pol II transcripts, among them some originating from essential genes, which may account for the observed toxicity. For RNAs synthesized by Pol I and Pol III we did not observe any alteration to steady-state levels upon Rho induction, although we cannot exclude a possibility of a non-detected small effect, due to the high stability of these RNAs.

The aforementioned mutant Rho-KE protein shows an intermediate growth defect phenotype compared to the one observed for the wild-type Rho expressed in yeast. By using this mutant protein we were able to search for synergistic sensitivity effects. Treatment of Rho-KE expressing cells with nucleotide-depleting drugs leads to aggravation of growth defect phenotype. Since it is known that these drugs lead to a decrease in rate and processivity of RNA synthesis, thus exacerbating transcription elongation defects, our results suggest that

Rho induces a growth defect due to its action on Pol II-dependent transcript elongation. This conclusion is supported by the finding that Pol II mutants defective in transcription elongation also aggravate the Rho-KE-induced mild growth defect. Likewise, Pol II mutants with an increased elongation rate not only alleviate the mild growth defect of Rho-KE, but also partially relieve the severe growth defect induced by Rho expression.

Among the Pol II transcripts tested, PMA1 showed the highest sensitivity with 60% to 70% reduction in the steady-state level. However, premature transcription termination was not the cause for this reduction since no prematurely terminated mRNAs could be detected. In an attempt to find out if the potentially early terminated transcripts are degraded, and thus escape detection, the exosome component Rrp6 was deleted. Surprisingly, this caused a rescue of the growth defect with concomitant restoration of transcript abundance. This result led to the conclusion that, following Rho action, the fully transcribed transcripts are recognized as aberrant and degraded by the nuclear exosome. Our hypothesis was that Rho induces toxicity and production of aberrant transcripts by tracking along the RNA and competing with the endogenous mRNA processing and packaging factors or even by displacing them during its translocation. Thus, we reasoned that the overexpression of some of these factors should alleviate the Rho-induced growth defect. In the screen of 40,000 colonies we isolated 19 positive candidates among which 13 genes whose proteins have relatively well-known or predicted molecular functions (ranging from transcription regulation to mRNA processing, packaging and export) and 6 with putative functions that are unrelated to mRNA metabolism or proteins with unknown molecular functions (Table 2). In summary, this study on the use of bacterial Rho factor in yeast represents a valuable approach in research of interplay between transcription, mRNP biogenesis and export.

Table 1.2. Dosage suppressors of the Rho-induced grovth defect[a] (Mosrin-Huaman et al., 2009)

Gene name and type of suppressor	Systematic name	Suppression phenotype	Rescue of PMA1 mRNA	Molecular function
Factors involved in transcription regulation, mRNA processing, and export				
TAF14	YPL129w	+++	+++	Subunit of TFIID, TFIIF, and mediator
RAD3	YER171w	+	–	DNA helicase involved in nucleotide excision repair and transcription
RAD26	YJR035w	+	–	Involved in transcription-coupled repair
RNA14	YMR061w	++	++	Component of CFIA
PCF11	YDR228c	++	++	Essential component of CFIA
SUB2	YDL084w	++	+	Component of the TREX complex required for nuclear mRNA export
MEX67	YPL169c	++	+++	RNA binding protein involved in nuclear mRNA export
MTR2	YKL186c	+	–	Mtr2p and Mex67p form a mRNA export complex that binds to RNA
MLP2	YIL149c	+++	+	Myosin-like protein associated with the nuclear envelope
CTL1[b]	YMR180c	+	+	mRNA triphosphatase-like capping enzyme
SXM1	YDR395w	++	+	Suppressor of mRNA export mutant
RNA1	YMR235c	++	++	GTPase-activating protein for Gsp1p, involved in nuclear transport
TIF3	YPR163c	++	+	Translation initiation factor eIF4B
Proteins with ill-defined or unknown molecular functions				
CCT7	YJL111w	++	–	Chaperone involved in de novo protein folding
RAX1	YOR301w	++	–	Protein involved in bud site selection during bipolar budding
KIN3	YAR018c	++	–	Protein kinase
PEX30	YLR324w	++	++	Peroxisomal membrane protein
ORF1	YDR520c	++	++	Putative transcription factor; contains the Zn(II)2Cys6 motif
ORF2	YIL152w	++	–	Q-rich domain protein

[a] +, ++, and +++ indicate the extent (weak, medium, and strong, respectively) of Rho-induced growth defect suppression. The symbols also pertain to the strength of PMA1 mRNA restoration; – indicates faint or absent PMA1 mRNA restoration. The effects were evaluated under stringent conditions of Rho expression (i.e., the $TetO_7$ promoter).
[b] Isolated twice during the screen.

Further research using Rho assay demonstrated the aforementioned Nrd1 involvement in Rho induced quality control and provided further insights into possible mechanism of Rho action on yeast transcripts (Honorine et al., 2011) (Figure 1.19). Likewise, a novel study contributes to better understanding of functional contribution of the exosome and its cofactors to this quality control mechanism (Stuparevic et al., 2013).

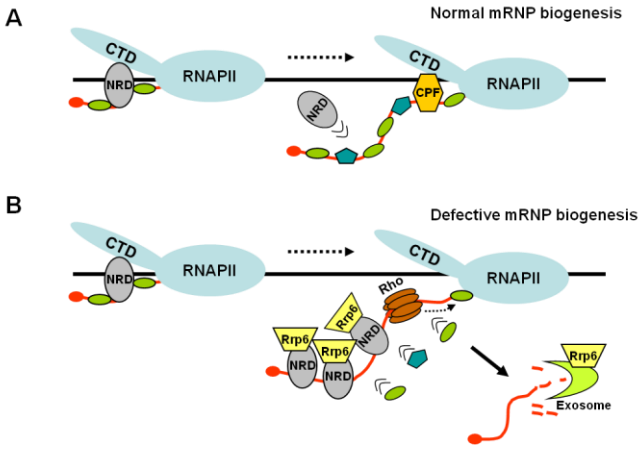


Figure 1.19: *Model of Rho-induced quality control mechanism and transcript degradation* (Honorine et al., 2011). (A) During normal mRNP biogenesis Nrd1 factor is recruited to the transcription site through interaction with the Ser5-P mark of Pol II CTD but does not stay bound long if its role in transcription termination or QC is not necessary. (B) By the proposed model of Rho-induced defective mRNP biogenesis, Rho would displace processing factors and RBPs which would uncover binding sites for Nrd1 to be recruited. This would signal the recruitment of the degradation machinery and after transcription termination and release of the aberrant transcript, the same would be degraded by Rrp6 exonuclease alone or in association with the whole exosome complex.

1.4 The research question

Transcription is a highly complex process fundamentally coupled to transcript processing and nuclear export in eukaryotes, thus ensuring fitness, accuracy and swiftness of the initial phase of eukaryotic gene expression. Key agents performing these functions are numerous protein factors recruited to the nascent transcript with a role to secure, process, verify and escort/transport mature transcripts into the cytoplasm for translation. Mutations and/or deletions of these factors lead to different phenotypes, readily observed on the transcript level. Previous research of mRNP and QC has mostly been done on a model system where the influence on a HSP104 transcript was observed under heat-shock conditions. Using this model a question arises whether the observed phenotypes are caused by the introduced mutations or are they partially or fully caused by the manipulations performed under heat-shock conditions.

To offer a different strategy in the research of mRNP biogenesis and quality control, previously known features and function of a bacterial transcription termination factor Rho were used in our laboratory group to produce aberrant mRNAs targeted by QC apparatus in yeast. The posed hypothesis was that Rho factor expressed in yeast could load the mRNA as in bacteria and by tracking in the 3' direction, it would interfere with normal deposition of mRNA processing and packaging factors, which in turn could lead to production of prematurely terminated, aberrantly processed and/or export incompetent transcripts. Research on this model is performed at room temperature conditions and initial studies carried out by our group show evidence of Rho induced production of aberrant mRNA transcripts, targeted by the Nrd1 governed nuclear QC and retained/degraded by the Rrp6 exonuclease, which is assisted by different cofactors of the RNA degradation machinery (Mosrin-Huaman et al., 2009; Honorine et al., 2011; Stuparevic et al., 2013).

This manuscript presents the research done to observe the influence of Rho factor on normal mRNP biogenesis. Of particular interest was the effect on the recruitment profile of THO-Sub2 subunits, a major factor in coupling transcription, mRNP biogenesis and export. The starting hypothesis was that Rho, as a powerful molecular motor, strips the nascent transcript of some key protein factors, among them presumably THO-Sub2 and thus renders transcripts defective. In addition, we show that Rrp6 exonucleolytic activity is the main degradation pathway for Rho induced aberrant transcripts.

2 MATERIALS AND METHODS

2.1 Yeast strains and plasmids

To obtain the RRP6-TAP strain in the genomic background of our choice BMA41, polymerase chain reaction (PCR) amplification was performed on template genomic DNA from the strain RRP6-TAP::URA obtained from Euroscarf (strain Sc1480). The strains were eventually screened by PCR and immunoblotting. For construction of Rrp6-Myc strain the PCR amplicon from pYM18 containing the Myc tag and kanamycin marker was used to transform BMA41 yeast strain.To construct the RRP6-Y361A strain, the PCR amplicon made on genomic DNA from strain RRP6-MYC (with the forward primer harboring the mutated codon) was used to transform BMA41 cells. The selected transformants were analyzed by PCR amplification and sequencing of the PCR products to confirm the presence of the mutated codon, and then immunoblotting was used to verify the presence of the tag. A PCR-based one-step deletion/tagging technique was used to create null mutant and strains expressing the various C-terminally tagged proteins with appropriate cassette plasmids and oligonucleotides (Janke et al., 2004). For construction of MFT1-Myc strain the PCR amplicon from pYM18 containing the Myc tag and kanamycin marker was used to transform W303 yeast strain. For THO2-Myc, HPR1-Myc and SUB2-Myc constructions, pYM19 was used as a cassette plasmid, giving PCR amplicons with the Myc tag and HIS3 marker used to transform BMA41 yeast strain. Plasmid pFA6a-kanMX4 was used as a template to obtain kanamycin marker bearing PCR amplicon used to transform BMA41 wt and a strain with SUB2-Myc to obtain *MFT1* deletion. For strains with tagged Tho2, Hpr1 and Thp2 combined with *MFT1* deletion, the *mft1Δ* strain was transformed with the aforementioned amplicon used to tag these proteins with Myc. The lists of yeast strains and plasmids used in our research are presented in tables 2.1 and 2.2, respectively.

2.2 Cell growth and Rho induction

S. cerevisiae cells were grown according to standard procedures at 25 °C in synthetic complete medium with glucose (2%) as a carbon source and with appropriate bases and amino acids omitted as necessary for selection. The cell growth was monitored by measuring the optical density at 600 nm. The expression of Rho from pCM185-Rho-NLS plasmid, when present, was repressed by growing the cells in the presence of doxycycline (Dox) at a final

concentration of 1 µg/mL, whereas the expression of Rho protein was obtained at 0.2 µg/mL Dox (RNA extraction for Northern blotting) or by omitting Dox from the growth medium (ChIP). To induce Rho, the cells harboring the expression plasmid were first pre-grown with 0.5 µg/mL of Dox (moderate expression) for 6-7 h. The cells were then washed extensively (three to four times) with medium lacking Dox, diluted, and allowed to grow overnight in the selective medium with low concentration or lacking Dox. After the overnight growth (14–16 h), the Rho-induced cells were typically at an optical density of 0.25-0.4, whereas the non-induced cells were at an optical density of around 0.8-1. To analyze the samples, the non-induced cultures were diluted with fresh medium to adjust the optical density to the induced cultures before harvesting the cells (protein and RNA extractions) or cross-linking (ChIP).

2.3 Serial dilutions test

Yeast strains transformed with the appropriate plasmids and grown overnight in Rho-repressing conditions were prepared as four 10-fold serial dilution samples. The samples were plated as 10 µL drops onto both Rho-repressing (1 µg/mL Dox) and Rho-inducing (no Dox) selective plates. Plates were grown for three days at 25˚C and then photographed.

2.4 RNA isolation and Northern blotting

Yeast cells transformed with the appropriate plasmids were grown with or without Rho induction, and total RNAs were extracted by the hot phenol method (Schmitt et al., 1990). Harvested cells were washed once with water, once with AE Buffer (50 mM sodium acetate pH 5.3, EDTA 10 mM pH 8.0) and resuspended in 400 µL of AE Buffer to which we added SDS to 1%, glass beads and vortexed. We added equivalent volume of acidic phenol-chloroform (5:1 pH 4.5) preheated to 65˚C. After three times of 1 min vortexing followed by a 1 min incubation at 65˚C, we put the samples in dry ice for 2 min and again repeated the previous step of heating and vortexing. We centrifuged the sample at 4˚C, took the upper, aqueous phase and repeated the extraction with the equivalent volume of phenol-chloroform-isoamyl alcohol (25:24:1) pH 6.7. On the obtained aqueous phase we added sodium acetate to final 0.3 M pH 5.3, three volumes of 100% EtOH and precipitated for 20 min at -80˚C. Samples were centrifuged for 15 min at 14 000g, the pellet was washed with 80% EtOH, dried shortly and resuspended in an

arbitrary amount of water. Samples were kept on ice, quantified with a Nanodrop spectrophotometer and their concentration was adjusted to 1.2 µg/mL.

For Northern blotting, the RNA samples were prepared by adding 10 µL of loading buffer (571µL/mL of formamide 99%, 203µL/mL of formaldehyde 37%, MOPS 1x, Northern blue 1x, ethidium bromide 0.05ng/mL; MOPS 10x – MOPS (3-(N-morpholino) propanesulfonic acid) 4%, sodium acetate 50 mM pH 5.2, EDTA 10 mM; Northern blue 10x – bromophenol blue 0.25%, glycerol 30%) on 10 µL of each sample (12 µg RNA). Samples were denatured for 3 min at 90˚C and placed on ice for 5 min. For RNA separation we used 1.2% agarose-formaldehyde-MOPS gel. After the run, the gel was washed two times in water and then in SSC 10x with mild agitation (SSC 20x – NaCl 3 M, Na-citrate 0.3 M). Transfer was carried out onto a nylon membrane (Amersham Hybond-XL) by capillary blotting in SSC 10x. After an over-night transfer, the membrane was washed shortly in SSC 2x and the RNA was fixed on the membrane by exposure to 240nm UV light, 0.120 Jouls/cm2 for 1min. Membrane was incubated 3h in preheated (45˚C) hybridization solution – Church Buffer (Na2HPO4 0.5M, EDTA 10mM, SDS 7%), at 45˚C, with agitation. After this prehybridization step, we removed the Church Buffer, added 15mL of fresh buffer containing 10ng/µL of the 5' end radioactively labeled oligonucleotide probes (Table 2.3) and incubated over-night at 45˚C, with agitation. After hybridization we washed the excess of the probe in SSC 2x, SDS 0.1% until radioactivity went down to 200 cpm. After membrane exposition to a PhosphoImager screen for a few hours, we washed all remaining radioactivity at 75˚C in Tris 10mM pH7.5, SDS 0.1%. Hybridization signals were quantified with a Storm 860 PhosphorImager (Molecular Dynamics) and the data were processed with ImageQuant software version 2005.

2.5 RT-PCR and RT-qPCR

RNAs were treated by DNase using an RTS DNase™ kit (Mo Bio Laboratories, Inc.) according to the instructions of the manufacturer. One microgram of total RNA was used in a Superscript II RT reaction (Invitrogen) (results in section 3.1) with the two gene-specific oligonucleotides in the same 25-µL reaction mixture (18 S and PMA1) or Maxima First Strand cDNA Synthesis kit for RT-qPCR (Fermentas) (results in section 3.2.2). The cDNA (1 µL) was PCR-amplified with specific oligonucleotides. To achieve linear range

amplifications, 20 cycles (PMA1 and 18 S cDNA) or 21 cycles (RRP6 and RRP47) were applied. The PCR products were analyzed by agarose gel electrophoresis. For real-time PCR quantifications, 100- to 1000-fold (results in section 3.1) or 20-fold (results in section 3.2.2) diluted cDNA was amplified in a Roche LightCycler 480 with the Maxima SYBR Green quantitative polymerase chain reaction (qPCR) Master Mix detection kit from Fermentas as recommended by the supplier. The qPCR data sets were analyzed using the ΔΔCt method, and the results were normalized to 18S rRNA RT-qPCR amplification, which was used as internal control. The level of PMA1 mRNA for each sample was expressed relative to the PMA1 mRNA abundance in wild-type BMA41 cells and in the absence of Rho, which was set as 1. Amplifications were done in duplicate for each sample, and three independent RNA extractions were analyzed.

2.6 Protein extraction and Western blotting

Whole-cell protein extracts were prepared as described in Kushnirov, (2000). Tested strains transformed with Rho-expressing plasmid were grown at 25˚C in either liquid medium or on plates containing 1 μg/mL of Dox (Rho repression) and without Dox (Rho induction). Cells were taken as an aliquot or scraped off the plate and resuspended in 100 μL of distilled water. We added 100 μL of 0,2M NaOH and incubated 5 min at room temperature. Samples were pelleted, resuspended in 50 μL of 2x sample buffer (10% glycerol, 140mM β-mercaptoethanol, 0.1% bromophenol blue, 1% SDS and 112mM Tris/HCl pH 6.8), boiled for 3 min at 95˚C and pelleted again. Samples were placed on ice and 10 μL of supernatant was loaded for gel electrophoresis in SDS-10% polyacrylamide gel.

Western blotting was performed according to standard procedures (Sambrook and Russel, 2001). Protein transfer onto a pvdf membrane was performed by semi-dry transfer method using Bjerrum&Schafer-Nielsen Transfer Buffer (48 mM Tris base, 39 mM glycine, 20% methanol and 0.037% SDS). For Blocking Buffer we used 5% milk in TBST (TBS 10x – 20mM Tris-HCl pH 7.5, 1.5M NaCl – diluted in 0.1% Tween 20) and the same solution was used for incubation of membrane with antibodies. Tagged proteins were detected with anti-Myc-HRP (Santa Cruz Biotechnology) or with peroxidase-anti-peroxidase (Sigma) for detection of protein A in TAP tag. Rho protein was detected with rabbit polyclonal anti-Rho antibodies (custom preparation from Eurogenetec). Tfs1 was detected with specific polyclonal

antibodies (provided by H. Benedetti). Secondary antibodies (goat anti-rabbit-HRP IgG, Promega) were used when necessary. Visualization on x-ray films was performed following enhanced chemiluminescence (Novex ECL Chemiluminescent Substrate Reagent).

2.7 Chromatin immunoprecipitation

Strains harboring appropriate C-terminal-tagged proteins and the "no tag" control strain (BMA41), coupled with the Rho expression plasmid, were grown in selective media with or without Dox as described in section 2.2. Forty milliliters of grown cells were fixed with 1% formaldehyde for 20 min. After glycine addition to stop the reaction at 0,4M final, the cells were washed first in 20 mM TRIS/HCl pH 8.0 buffer, then in ChIP1 buffer (50 mM HEPES/NaOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% TRITON, 0.1% DOC, 0.05% SDS) and lysed with glass beads in 500 µL + 500 µL ChIP1 buffer with 0.5 mM Pefabloc and 40 u/mL of RNasin (Promega) to isolate chromatin. The cross-linked chromatin was pelleted, resuspended in 1.6 mL of the same buffer and sheared by sonication to reduce average fragment size to ~500 bp, with samples kept on ice. Chromatin fractions of 100 µL were taken for total cell extract (input) and 400 µL for each immunoprecipitation reaction (output). For RNase sensitivity assay we omitted the RNasin from the buffer in which sonication was performed onwards and added 15µL of RNase A, T1 mix (Fermentas) to treated output samples and 15 µL of the appropriate buffer to the control input sample. Immunoprecipitation in output samples was made with 40 µL of IgG Sepharose (GE Healthcare), protein G-Plus-agarose premixed with anti-Myc antibodies (sc-40 X, Santa Cruz Biotechnology), or protein G-Plus-agarose premixed with anti-RNA polymerase II antibodies (8WG16, Covance). After overnight incubation at 4 °C, beads were washed extensively, first with ChIP2 buffer (50 mM HEPES/NaOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% TRITON, 0.1% DOC, 0.05% SDS), then with LiCl buffer (10 mM TRIS/HCl pH 8.0, 0.25 M LiCl, 1mM EDTA, 0.5% Nonidet P40, 0.5% DOC) and finally with TE buffer. Chromatin was eluted with 140 µL of 1x decrosslink buffer (5x: 125 mM TRIS/HCl pH 7.5, 25 mM EDTA, 2.5% SDS). Eluted supernatants (output) and the input controls were hydrolyzed with Pronase (0.8 mg/ml final concentration, Sigma) during an overnight incubation at 65 °C to reverse cross-linked DNA complexes, after which 5 µL of RNase was added to each sample and incubated 30 min at 52˚C. DNA was extracted using the Qiagen PCR cleanup columns, eluted with 50 µL of TE buffer. The immunoprecipitated DNAs (output) were quantitated by real-time PCR

(LightCycler 480, Roche, with the products and instructions recommended by the supplier) using all or some of the six sets of primers located along the PMA1 gene (Fig. 3.1; Table 2.4) and normalized to a 1:200 dilution of input DNA. Amplifications were done in duplicate for each sample, and averages and S.D. were calculated on the basis of at least two independent experiments.

2.8 RNA- Fluorescence in situ hybridization (FISH)

Yeast cells harboring the Rho expression plasmid were grown under repressing (+ Doxy) or inducing (- Doxy) conditions in selective medium containing adenine (100 ng/ml) for 16 h as described in section 2.1. Cells were collected at low log-phase (OD_{600} ~ 0.3) and were prepared for analysis as described in Kallehauge et al., (2012). Cells were fixed by adding 32% (v/v) formaldehyde directly to the media to a final concentration of 4% (v/v) for 45 min at room temperature (20–25 °C). The cell wall was digested with lyticase, cells were attached to poly-L-lysine-coated coverslips and stored in 70% (v/v) ethanol at 4 °C. Before hybridization, cells were rehydrated twice in 1x SSC for 5 min and once in 10% (v/v) formamide and 1x SSC (5 min). Coverslips were inverted onto hybridization solution containing oligo $d(T)_{40}$ labeled with Cy3 (Amersham) and hybridized according to manufacturer's instructions. Fluorescent images acquired (as in Kallehauge et al., (2012) with a Leica DM6000 epifluorescence wide field microscope equipped with a Coolsnap HQ2 camera and a Leica 100X PL APO 1.4-0.7 oil NA objective) were analyzed with the MetaMorph software.

Table2.1: *Saccharomyces cerevisiae* strains used in this study

Strain name	Genotype	Source
BMA41	*MATa ade2-1 ura3-1 leu2-3,112 his3-11,15 trpΔ can1-100*	Baudin-Baillieu et al., (1997)
W303	*MATa ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100*	Thomas and Rothstein, (1989)
rrp6Δ	as BMA41 with *rrp6Δ::KAN4*	Mosrin-Huaman et al., (2009)
Rrp6-Myc	as BMA41 with *Rrp6-MYC::KAN*	Stuparevic et al., (2014)
Rrp6-Y361A	as RRP6-MYC with *Rrp6-Y361A*	Stuparevic et al., (2014)
DLY1354	as W303 with *dis3D::KAN [pBS3269-DIS3-TEV-PROTA, LEU2]*	Lebreton et al., (2008) and D. Libri lab
DLY1350	as W303 with *dis3D::KAN [pBS3278-dis3$_{D171N}$-TEV-PROTA, LEU2]*	Lebreton et al., (2008) and D. Libri lab
DLY1358	as W303 with *dis3D::KAN [pBS3270-dis3$_{D551N}$-TEV-PROTA, LEU2]*	Lebreton et al., (2008) and D. Libri lab
Rrp6-TAP	as BMA41 with *Rrp6-TAP::URA3* or *HIS* or *KAN*	Stuparevic et al., (2014)
Rrp4-TAP	as W303 with *Rrp4-TAP::HIS*	D. Libri laboratory
Rrp41-TAP	as W303 with *Rrp41-TAP::HIS*	D. Libri laboratory
Tho2-Myc	as BMA41 with *THO2-Myc::HIS3*	This study
Hpr1-Myc	as BMA41 with *HPR1-Myc::HIS3*	This study
Mft1-Myc	as W303 (*MATa ade2-1 ura3-1 leu2-3,112 his3-11,15 trp1-1 can1-100*) with *MFT1-Myc::KAN*	This study
Thp2-Myc	as BMA41 with *THP2-Myc::HIS3*	This study
Sub2-Myc	as BMA41 with *SUB2-Myc::HIS3*	This study
mft1Δ	as BMA41 with *mft1Δ::KAN*	This study
mft1Δ Tho2-Myc	as *mft1Δ* with *THO2-Myc::HIS3*	This study
mft1Δ Hpr1-Myc	as *mft1Δ* with *HPR1-Myc::HIS3*	This study
mft1Δ Sub2-Myc	as Sub2-Myc with *mft1Δ::KAN*	This study

Table2.2: Plasmids used in this study

Plasmid name	Yeast genes	Rho expression	Backbone vector	Source
pCM185	*CEN TRP1*			Euroscarf
pCM185-Rho-NLS	*CEN TRP1*	pTetO$_7$::*Rho-NLS*	pCM185	Mosrin-Huaman et al., (2009)

Table2.3: Oligonucleotides used for Northern blot, Reverse Transcription and RT-qPCR

Targets	Assay	Amplicon	Oligonucleotide sequence	
			Forward	**Reverse**
18S	NB			5’ GGTTAAGGTCTCGTT 3’
	RT			5’ AGGTTAAGGTCTCGTTCG 3’
	RT-qPCR	707	5’-GAACTTTGGGCCCGGTTG 3’	5’ AGGTTAAGGTCTCGTTCG 3’
		197	5’ TTGACGGAAGGGCACCACCA 3’	
RRP6	RT			5’ GGGGACCACTTTGTACAAGAAAGCTGGGTCTTTTGCGACATTTCTTGATTCATAAA 3’
	RT-qPCR	451	5’ GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACCCATGACTTCTGAAAATCCGGATGTA 3’	5’ GGGGACCACTTTGTACAAGAAAGCTGGGTCGACTCTTTTGGTGGGAGAAAAATTCTT 3’
RRP47	RT			5’ CTACTTCTTCCCTCCTTTC 3'
	RT-qPCR	377	5’ CAAAGGCGTTAGACGAGCTG 3'	5’ CGTATGCTTCCCTTGAAAGT 3'
PMA1	NB			5’ CAATAGCATCCAAACCCTTCTTC 3’
	RT			5’ GGAATTTCAACCAATTGACCGTC 3’
	RT-qPCR	240	5’ CAATCTAATCACGGTGTCGACGACGAAGAC 3’	5’ GGCTTCCATAACGAATTGAATTGGACCG 3’

Table2.4: PMA1 primers used in ChIP experiments

Alias	Name	Sequence
5’UTR	F -666	5’ TGTATTTCCTAATGCGGCACT 3’
	R -666	5’ CCCGAAAGGCATATGGATAACAT 3’
5’end	F 151	5’ CAATCTAATCACGGTGTCGACGACGAAGAC 3’
	R 390	5' GGCTTCCATAACGAATTGAATTGGACCGAC 3'
middle	F 1010	5’ GTTTGCCAGCTGTCGTTACCACCAC 3’
	R 1235	5’ GCAGCCAAACAAGCAGTCAACATCAAG 3’
3’end	F 2251	5' AACCTACCAAGATTATGGGGTATGTC 3’
	R 2532	5' CCAACCGAATAAGGTAAACATGGTAGCGATG 3’
3’UTR-1	F 3287	5’ GAAAATATTTGGTATCTTTGCAAGATG 3’
	R 3500	5’ GTAAATTTGTATACGTTCATGTAAGTG 3’
3’UTR-2	F 3671	5’ AAGAAAGCAAACAAATCGCCAG 3’
	R 3671	5’ TCATCTAGAGTAATGACGCCTTAGT 3’

3 RESULTS

3.1 Nature of Rho-induced transcript degradation

Les transcrits aberrants produits par l'action de Rho sont dégradés par la machinerie de dégradation Rrp6-dépendante. Pour déterminer l'importance de Rrp6 dans ce processus de dégradation, nous avons généré un mutant Rrp6 catalytiquement inactif. Nous cherchions également à démontrer l'importance de l'implication des autres composantes de la machinerie de dégradation nucléaire dans ce processus de dégradation. Les résultats présentés dans cette section n'excluent pas un rôle possible de Dis3 et du cœur de l'exosome, mais confirment bel et bien le modèle stipulant que la dégradation des transcrits induite par Rho est causée par l'activité exonucléase de Rrp6.

The newly implemented assay in our laboratory group to study nuclear QC in *S. cerevisiae* relies on a heterologous expression of the bacterial Rho factor, which perturbs normal mRNP biogenesis using its RNA-dependent helicase/translocase activity. Rho action in this system induces the production of full-length transcripts, nevertheless recognized as aberrant and degraded by the Rrp6-dependent degradation machinery (Mosrin-Huaman et al., 2009). The model transcript used in our research using Rho factor is the product of a constitutive gene *PMA1*. This transcript shows high sensitivity to Rho action with a 60% to 70% reduction in the steady-state level in *S. cerevisiae*. By ChIP analysis of Pol II distribution in the presence and absence of Rho, we show that this reduction is a consequence of Rho action on the transcript and not a result of a transcriptional defect that would cause premature transcription termination (Figure 3.1).

In our previous publication we have shown that targeting and degradation of the Rho-induced aberrant transcripts is mediated by co-transcriptional recruitment of the RNA-binding transcription termination factor Nrd1 and the exosome component Rrp6 (Honorine et al., 2011). Next, we wanted to determine the extent of involvement in the Rho-induced transcript degradation of the two catalytically active components of the exosome, Rrp6 and Dis3.

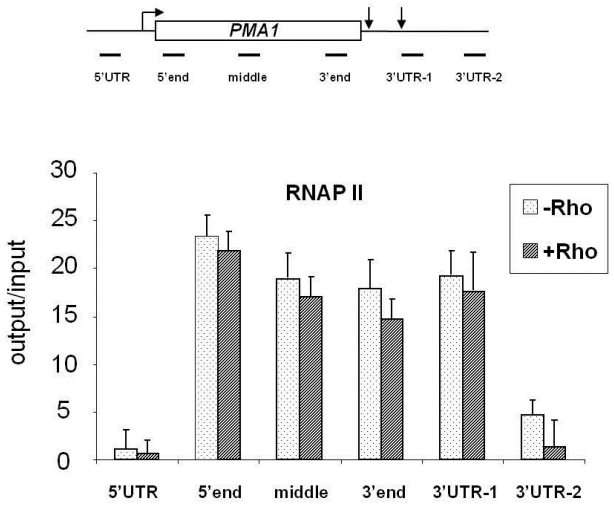


Figure 3.1: *Rho factor expressed in yeast does not significantly interfere with transcription by RNA polymerase II*. Quantification by real-time PCR of ChIP samples obtained from BMA41 yeast strain harboring Rho expression vector (pCM185-Rho-NLS) and grown either in repressing (-*Rho*) or inducing (+*Rho*) conditions. Immunoprecipitation was performed using specific Pol II antibodies 8WG16 and six sets of oligonucleotides were used in quantification by qPCR as represented schematically in the upper panel. The average of three independent experiments is shown where immunoprecipitated (output) samples were normalized to input following quantification by real-time PCR. Arrows in the upper figure indicate positions of transcription start site and poly(A) sites, while error bars in the graph represent standard deviations (S.D.).

3.1.1 Degradation of transcripts by Rrp6 in Rho expression system

Doxycycline-regulated expression of Rho-NLS from a plasmid vector in yeast leads to specific Rho-induced phenotype. Wild type yeast strain with an activated Rho expression system (-Dox) shows severe growth defect phenotype in comparison with the strain bearing an inactive Rho expression system (+Dox) (Figure 3.2A). Together with this growth defect, by using Northern blot analysis, we can observe on the molecular level a large decrease in the steady-state level of a model transcript PMA1 (Figure 3.2B). A detailed study of transcripts affected by Rho shows that they have a normal length and poly(A) tails but are nevertheless targeted as aberrant and degraded by the nuclear QC system (Mosrin-Huaman et al., 2009; Honorine et al., 2011). The same study determined the involvement of the exosome component Rrp6 in this Rho-induced transcript degradation. Deletion of *RRP6* leads to a relief of growth defect in strains with expressed Rho factor, as well as a rescue of PMA1

steady-state level (Figure 3.2). To further strengthen the importance and involvement of Rrp6 exonuclease in this transcript degradation pathway, we constructed a catalytically inactive Rrp6 with *Y361A* mutation in the chromosomal locus, which leads to Rrp6 inactivation *in vitro* and *in vivo* (Phillips and Butler, 2003). Growth phenotype and Northern blot analysis show similar levels of suppression compared to the *rrp6Δ* strain (Figure 3.2). RT-PCR results confirm transcript degradation in wt strain with Rho expression, while we can clearly see the steady-state level of PMA1 is restored in both *rrp6Δ* and *rrp6-Y361A* (Figure 3.2C). These results confirm that Rrp6 catalytic activity is essential in elimination of Rho-induced aberrant transcripts.

Co-transcriptional recruitment of Nrd1 and Rrp6 along *PMA1* was previously revealed by ChIP assay in strains with Rho expression system (Honorine et al., 2011). This result endorses the conclusion of Rrp6 involvement in Rho-induced targeting and elimination of defective transcripts. To confirm that the observed relief of Rho-induced growth defect and transcript degradation in *rrp6-Y361A* strain is a direct result of the lack of catalytic activity in this mutant and not caused by a possible lack of transcription site recruitment of this mutant protein, we performed ChIP assay on Myc-tagged Rrp6-wt and Rrp6-Y361A coupled with Rho expression system. In Figure 3.2D we can clearly see a similar recruitment increase for both Rrp6 and Rrp6-Y361A under Rho induction conditions, compared to the controls with no tag or without Rho induction. Results presented in this section confirm the model which states that Rho-induced transcript degradation is caused by Rrp6 exonuclease activity.

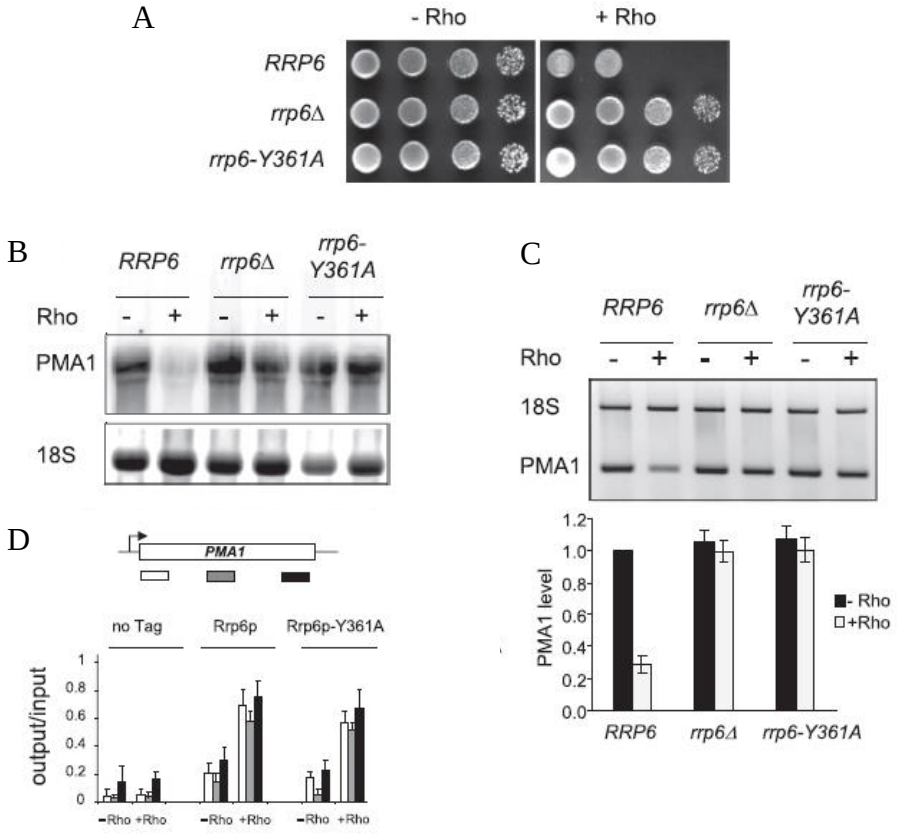


Figure 3.2: *Exonuclease activity of Rrp6 is necessary for degradation of Rho-induced aberrant transcripts.* (A) A 10-fold serial dilution test on BMA41 wt strain (*RRP6*), *rrp6Δ* mutant and a strain harboring the catalytically inactive Rrp6 (*rrp6-Y361A*), all of which were transformed with the Rho expression plasmid pCM185-Rho-NLS. Strains were grown in selective liquid medium under non-inducing conditions (1 µg/mL Dox) and spotted onto Rho-repressing (+Dox) or Rho-inducing (-Dox) plates and left to grow for three days at 25˚C. (B) Northern blot assay performed on the same strains as in (A). Total RNA was extracted from strains grown under Rho-repressing (1 µg/mL Dox) or Rho-inducing (without Dox) conditions. RNA samples (12 µg) were fractionated on an agarose gel and transferred to a membrane. Steady-state levels of PMA1 transcript were detected by a 5' end-labeled probe. The loading control 18S rRNA was visualized by ethidium bromide staining of the gel before membrane transfer. (C) Semiquantitative RT-PCR analysis performed on total RNA (1 µg) samples from strains as in (A), (B). Visualization was made using specific primers for PMA1 mRNA and 18S rRNA and ethidium bromide staining. Histogram shows PMA1 abundance relative to 18S rRNA as determined by quantitative RT-PCR. PMA1 signal for BMA41 wt strain in absence of Rho-expression is set to 1 as a reference value. The average of three independent experiments is shown, with error bars representing S.D. (D) Quantification by real-time PCR of ChIP samples obtained from yeast wt strain (*no Tag*) or strains harboring Myc-tagged Rrp6 variants, grown either in repressing (*-Rho*) or inducing (*+Rho*) conditions. Immunoprecipitated (*output*) samples were normalized to input as in Fig. 3.1 for regions 5', middle and 3' as represented schematically. The average of three independent experiments is shown, with error bars representing S.D.

3.1.2 The role of Dis3 and the exosome in Rho-induced transcript degradation

As described in the Introduction section of this manuscript, the catalytically inactive core exosome is associated with two components bearing catalytical activity, Rrp6 and Dis3. To fully affirm the proposed essential nature of Rrp6 exonuclease activity in degradation of Rho-induced aberrant transcripts, we sought to assess the involvement of the second catalytically active exosome component, Dis3. Since Dis3 is an essential factor in yeast and thus cannot be deleted, we took advantage of two separate mutant strains bearing a mutation either in the exonuclease or endonuclease activity. This allowed us to test separately each nuclease function of Dis3 by introducing the Rho expression system into the mutant strains. In *dis3-D551N* strain the exonuclease activity is disrupted (*exo*$^{-}$) while the *dis3-D171N* strain has no endonuclease activity (*endo*$^{-}$). As before, we first performed a growth test to see the possible influence of these mutations on Rho induced growth defect. In this assay we can clearly see that the growth defect in these two *dis3* mutants is comparable to the one in the wt strain with Rho expression and there is no growth rescue as in *rrp6Δ* strain (Figure 3.3A). In accordance with these results, in the RT-PCR experiment there is no significant transcript rescue in these two *dis3* mutants under Rho inducing conditions (Figure 3.3B). However, there is a slight recovery (10%) of the steady-state level of PMA1 transcript in the *endo*$^{-}$ mutant under Rho inducing conditions, which suggests a possible minor role for Dis3 endonuclease activity in Rho-induced degradation of transcripts (Figure 3.3B). Still, with these results we can conclude that Rrp6 exonuclease activity is the main hydrolytic activity involved in degradation of Rho-induced aberrant transcripts.

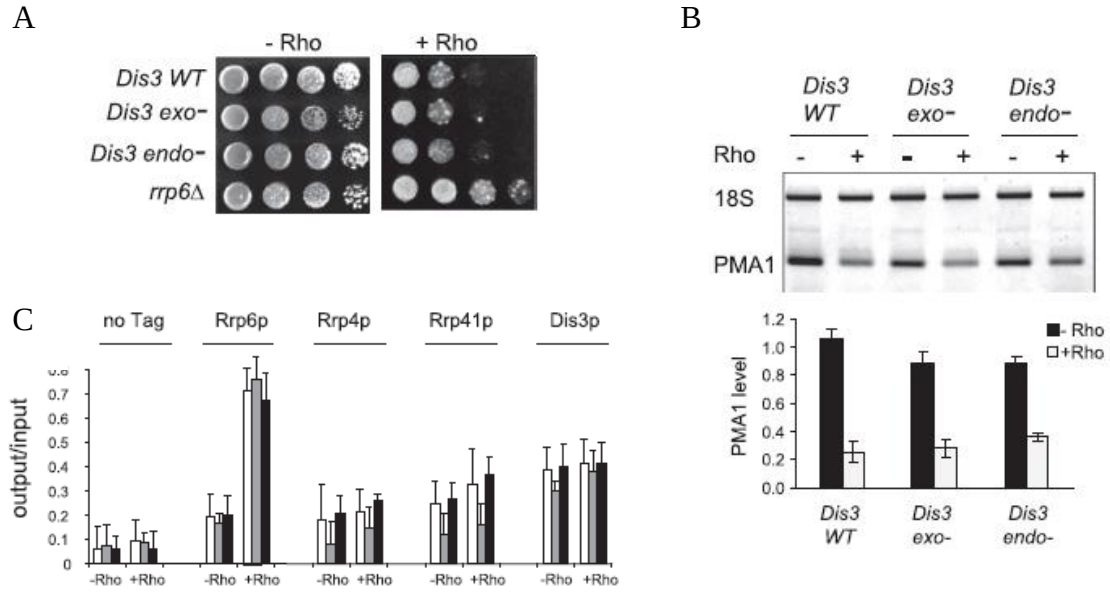


Figure 3.3: *A minor role of the core exosome and Dis3 in elimination of Rho-induced aberrant mRNPs*. (A) Serial dilution test performed as described in Fig. 3.2A on strains with mutations in Dis3 affecting its exonuclease (*exo*-) or endonuclease (*endo*-) activities together with control strains BMA41 wt and *rrp6Δ*, all transformed with the plasmid expressing Rho (pCM185-Rho-NLS). (B) Semiquantitative RT-PCR analysis on BMA41 and Dis3 mutant strains and real-time RT-PCR measurements performed as in Fig 3.2C. (C) Quantification by real-time PCR of ChIP samples obtained from yeast wt strain (*no Tag*) or strains harboring TAP-tagged core exosome subunits (*Rrp4* and *Rrp41*) or exosome cofactors (*Rrp6* and *Dis3*), grown either in repressing (*-Rho*) or inducing (*+Rho*) conditions. Immunoprecipitated (*output*) samples were normalized to input as in Fig. 3.2 (5', middle and 3' regions). The average of three independent experiments is shown, with error bars representing S.D.

Next, we wanted to see if there was any Rho-dependent increase or decrease in Dis3 recruitment to the transcription site. We also decided to examine the recruitment profile of two core exosome subunits, Rrp4 and Rrp41. For this purpose we used tandem affinity purification (TAP) tagged versions of the target proteins and performed ChIP experiment on strains with and without an activated Rho expression system. In parallel, we performed ChIP on Rrp6-TAP tagged strain coupled with Rho expression system, to compare the recruitment profiles and use as a positive control for other exosome components. According to the obtained ChIP recruitment profile of Dis3 protein, we can see a certain level of recruitment to the transcription site (Figure 3.3C). However, the level of recruitment is similar in the absence or presence of Rho expression, discarding the notion of a Rho dependent recruitment. Similar

results were obtained for the two members of the core exosome complex, with no noticeable dependence on Rho in recruitment to the transcription site. The lack of dependence on Rho for these three essential components of the exosome is clearly visible in comparison to the significant increase in recruitment of Rrp6 protein under Rho expression. As a control of a possible secondary Rho effect in these experiments, we performed Western blot assay on strains used in these ChIP experiments. Obtained results indicate that cellular protein levels of Dis3, Rrp4, Rrp41, as well as Rrp6, are not influenced by Rho expression (Figure 3.4).

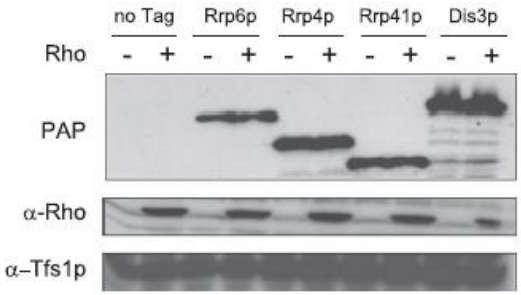


Figure 3.4: *Protein levels of exosome subunits are not influenced by Rho expression*. Western blot analyses of whole protein extracts isolated from wt (no Tag) or strains harboring TAP-tagged core exosome subunits (*Rrp4* and *Rrp41*) or exosome cofactors (*Rrp6* and *Dis3*), grown under Rho-inducing or repressing conditions. The extracts were fractioned by SDS-PAGE, transferred onto a membrane and detected using peroxidase-anti-peroxidase (*PAP*). Levels of Rho protein were visualized using specific polyclonal anti-Rho antibodies (*α-Rho*) and the loading control, yeast protein Tfs1 with functions unrelated to RNA metabolism, also using specific antibodies (*α-Tfs1*).

Expression of Rho factor in yeast strains leads to a sever growth defect phenotype coupled with Rrp6 dependent transcript degradation. Taken together, obtained results regarding the involvement of different components of the nuclear exosome complex in this process cannot discard a possible role of Dis3 and the core exosome, but do firmly confirm the role of Rrp6 as the main factor for degradation of aberrant transcripts in the presence of Rho.

3.2 Nature of Rho-induced transcript aberration

The initial research of Rho expression system developed in our group and its effect on yeast cells determined a dose-dependent toxicity observed on the phenotypic and transcript level (Mosrin-Huaman et al., 2009). Steady state-level of PMA1 transcript is severely reduced in the presence of Rho and this transcript loss results from Rrp6 driven degradation. Indeed, we showed in previous sections that catalytic activity of Rrp6 exonuclease is the main actor in transcript degradation process under conditions of Rho expression. The set model was that Rho action strips off the nascent transcript some crucial protein factors involved in mRNP biogenesis, which makes the transcript recognized as defective and susceptible to degradation.

TREX is a versatile complex involved in different steps of mRNP biogenesis. This complex has an important role in coupling different processes during transcription and contributes to speed and efficiency of mRNP biogenesis. Due to these characteristics, it is easy to envisage that perturbation of this complex would disrupt mRNP biogenesis and activate nuclear QC mechanisms. Indeed, deletion mutants of single THO-Sub2 members are most regularly used in the research of transcription QC mechanisms (Fasken and Corbett, 2009; Schmid and Jensen, 2010, 2013). With this information in mind, we decided to study THO-Sub2 members coupled with Rho expression system in an attempt to reveal the nature of Rho action in producing transcripts recognized as aberrant by the nuclear QC.

3.2.1 Rho and the THO-Sub2 complex

Afin de déterminer de quelle manière l'action de Rho peut affecter les protéines d'assemblage des mRNPs, ainsi que leur emplacement sur le transcrit naissant, nous avons effectué des expériences d'immunoprécipitation de la chromatine (ChIP) sur des souches dont les membres de THO-Sub2 étaient marqués avec étiquette Myc, couplées avec un système d'expression Rho. L'action de Rho interfère avec la stabilité du complexe THO et il est intéressant de noter que nous observons des profils de signaux ChIP différents entre les membres de ce complexe. En utilisant de la RNase dans les expériences de ChIP avec les souches contenant le système d'expression Rho, nous observons la dépendance à l'ARN du recrutement du complexe THO, ce qui avait été impossible à démontrer précédemment dans la souche sauvage.

3.2.1.1 Effect of Rho expression in strains with tagged THO-Sub2 members

According to our hypothesis, the members of THO-Sub2 complex are stripped off the nascent transcript by Rho helicase/translocase activity. To test this hypothesis, we decided to use ChIP assay to analyze Rho effect on the recruitment of THO-Sub2 members along the *PMA1* gene. To carry out ChIP experiment we decided to tag the THO-Sub2 members with a Myc epitope within the chromosomal locus. Before proceeding with ChIP experiment, we needed to verify that the introduction of a Myc tag does not disturb the Rho induced phenotype in the constructed stains. In the absence of a transcription repressor (Dox) Rho expression leads to a severe growth defect in comparison to the growth in the presence of Dox or in the strains bearing an empty vector. To confirm that the introduction of a Myc tag to the proteins of interest does not modify the Rho growth defect in yeast, we performed a serial dilution test on selective growth media in the absence (Rho expression) or presence (no Rho expression) of Dox. This assay was performed for wt and strains harboring a Myc-tagged protein of interest in the presence of an empty pCM185 vector or the pCM185-Rho-NLS Rho expression vector, as described in Materials and Methods, section 2.3. Growth of all tested strains harboring a Myc-tagged protein is impaired to the same extent as the wt strain under Rho expression (Figure 3.5).

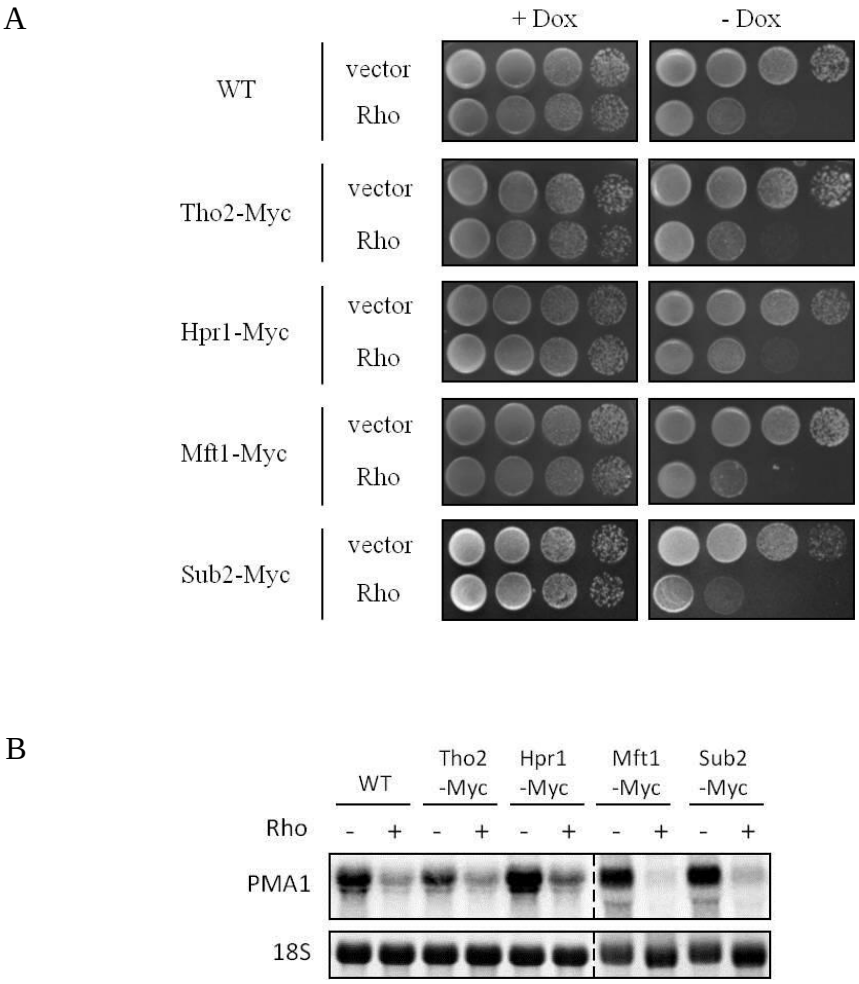


Figure 3.5: *Presence of a Myc tag on subunits of THO-Sub2 complex does not influence Rho-induced growth defect or transcript degradation*. (A) Serial dilution test performed as described in Fig. 3.2A on strains with a C-terminal Myc tag on the subunits of THO-Sub2 complex and BMA41 wt, all transformed either with a plasmid without Rho expression system (pCM185) or the plasmid expressing Rho (pCM185-Rho-NLS). (B) Northern blot analyses of total RNA extracts from strains with Myc-tagged members of THO-Sub2 complex grown in Rho-repressing or inducing conditions, as in Fig. 3.2B.

Likewise, Rho expression in yeast was shown to affect the steady-state levels of several mRNAs, with the highest impact on an essential PMA1 transcript (Mosrin-Huaman et al., 2009). Northern blot assay (described in section 2.4) was performed to confirm that the presence of a Myc tag does not influence the reduction in steady-state level of the PMA1 transcript under Rho expression. The effect of Rho expression on PMA1 accumulation is similar in wt and all constructed strains with the tagged protein of interest (Figure 3.5B).

To address the possibility of Rho indirect effect on proteins of interest on the cellular level, we compared the protein levels in the presence or absence of Rho expression. Western blot (described in section 2.6) was performed for constructed tagged strains and results show no significant change in protein levels dependent on Rho presence (Figure 3.7).

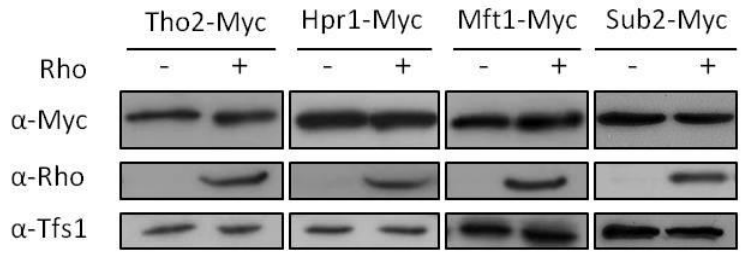


Figure 3.7: *Protein levels of THO-Sub2 subunits are not influenced by Rho expression*. Western blot analyses of whole protein extracts isolated from strains harboring Myc-tagged THO-Sub2 subunits, grown under Rho-inducing or repressing conditions and detected with anti-Myc antibodies (*α-Myc*). The experiment was performed and Rho and Tfs1 were detected as in Fig. 3.4.

3.2.1.2 ChIP of THO-Sub2 tagged strains in Rho expression system

To determine in which way Rho action might affect mRNP assembly proteins and their occupancy on the nascent transcript, we performed ChIP assay on the strains with Myc tagged THO-Sub2 members harboring an empty or the Rho expression vector under Rho inducing conditions (method described in section 2.7). THO-Sub2 cross-linking across the *PMA1* gene and the neighboring UTR was assessed by quantitative PCR (qPCR) using five corresponding primer-pairs (Figure 3.1A).

In Figure 3.8 we can see that ChIP distribution patterns in the absence of Rho are consistent with previous reports for all tested proteins (Strasser et al., 2002; Zenklusen et al., 2002; Abruzzi et al., 2004; Pfeiffer et al., 2013), with increased presence towards the 3' end of the *PMA1* gene. Interestingly, the expected displacement of mRNP proteins by Rho action is observed for only one tested protein, THO subunit Mft1 (Figure 3.8C). Other members of THO complex, as well as Sub2, actually show increased ChIP signals across the *PMA1* locus

under Rho inducing conditions (Figure 3.8A,B,D). Still, for all observed proteins, the attenuated or enhanced ChIP signals in the presence of Rho display an increase towards the 3' end comparable to the one in the absence of Rho.

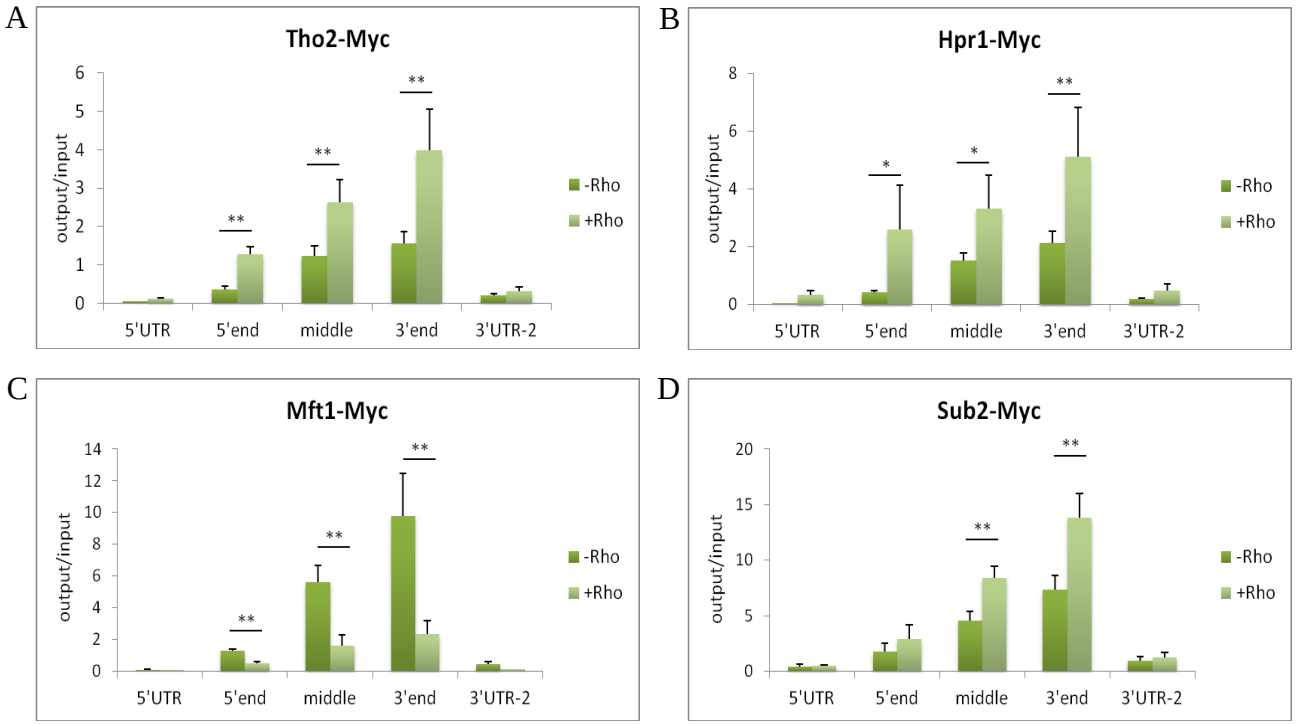


Figure 3.8: *Rho action influences recruitment of different THO-Sub2 members in different ways*. Quantification of the fold enrichment for PMA1 DNA in yeast strains with a Myc tag on (A) Tho2, (B) Hpr1, (C) Mft1 and (D) Sub2 proteins harboring Rho expression vector (pCM185-Rho-NLS) and grown either in repressing (*-Rho*) or inducing (*+Rho*) conditions. ChIP experiment was performed as in Fig. 3.1 using oligonucleotides as presented in schematic panel in Fig. 3.1. The average of at least four independent experiments is shown. Error bars in the graph represent standard deviation (S.D.) while asterisks denote the calculated p-value ** $p<0.01$, * $p<0.05$.

The obtained ChIP results are not caused by a transcriptional defect, nor by the change of cellular protein levels, as revealed by previously presented ChIP analyses of Pol II distribution and determination of cellular protein levels by Western blotting in the presence or absence of Rho (Stuparevic et al., 2013; section 3.1). Hence, Rho action disturbs the stability of THO complex which we observe by different ChIP signal trends between members of this complex. Different behavior of Mft1 compared to other TREX members evokes interest in the role and function of this ill-defined factor.

3.2.1.3 RNase sensitivity of THO-Sub2 in Rho expression system

At the beginning of this project, while performing ChIP experiments described in previous sections, we noticed variations in results from one experiment to another. These variations were greatly reduced by performing the experiment in an RNase-free environment, combined with the use of an RNase inhibitor. Interestingly, this occurrence was more pronounced in samples with the presence of Rho expression system then in samples without this system.

As previously mentioned (section 1.1.3.1.1), published ChIP results of THO subunits recruitment to chromatin after RNase treatment show no dependence on the growing nascent RNA chain (Abruzzi et al., 2004; Pena et al., 2012). To further explore the phenomenon observed during our experiments, we incubated the output samples with an RNase mix prior to immunoprecipitation. For the biggest THO complex subunit Tho2, without Rho expression, our results are in accordance with previously published results, hence we found no RNA dependence (Figure 3.9A). However, in the presence of an activated Rho expression system we see a large decrease in Tho2 recruitment to chromatin upon RNase treatment (Figure 3.9B, C). With RNase treatment Tho2 recruitment level goes down to less than 30% of the recruitment level in Rho expression system without RNase treatment. The same results are obtained for the two gene loci tested, the middle and the 3' end of *PMA1*.

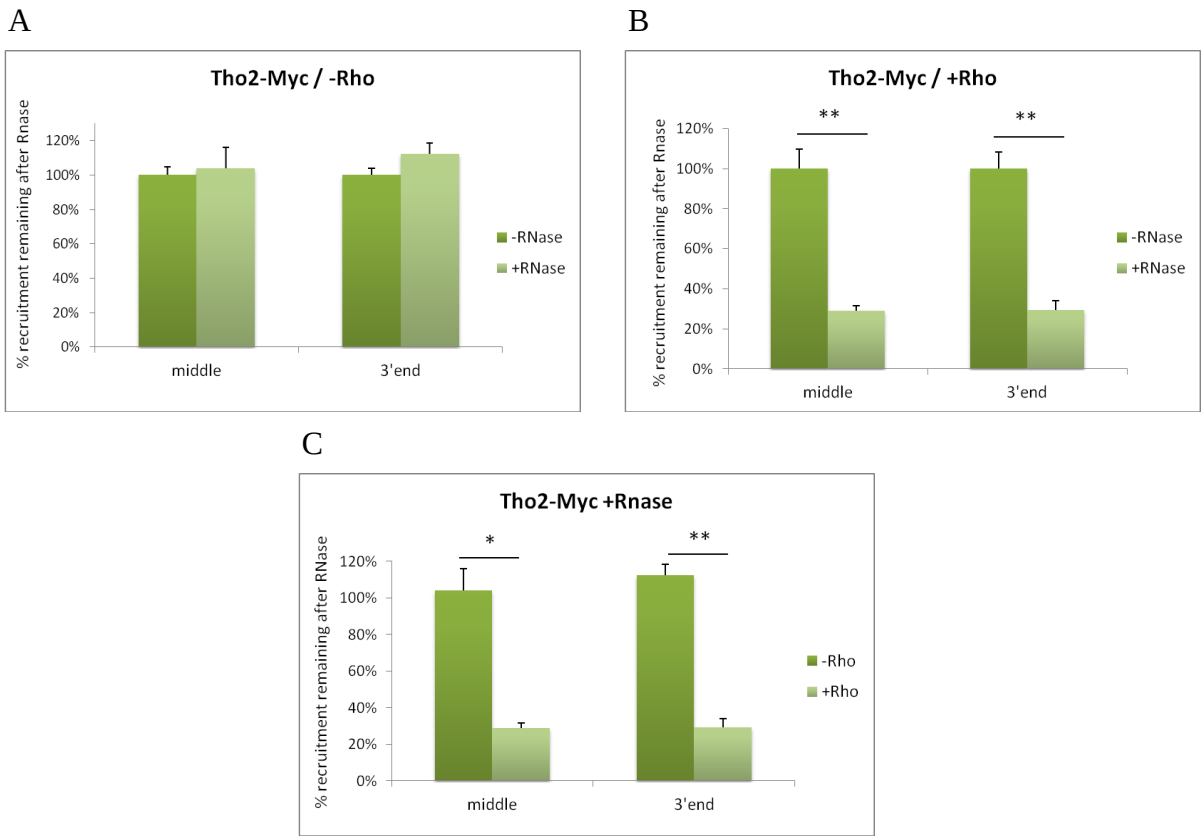


Figure 3.9: *Rho action reveals RNase sensitivity of Tho2 recruitment to transcription site*. (A) ChIP results for Myc-tagged Tho2 strain harboring an empty vector pCM185, with and without RNase treatment. The average result for samples untreated by RNase is set as a reference at 100% for each gene locus (middle and 3'end). Percentage after RNase treatment was calculated relative to the untreated samples. (B) ChIP results for Myc-tagged Tho2 strain harboring Rho expression vector pCM185-Rho-NLS, with and without RNase treatment. Results presented as in (A). (C) Comparison of percentages of Tho2 recruitment after RNase treatment relative to the untreated samples, without (*-Rho*) and with *(+Rho*) Rho expression. ChIP experiment was performed as in Fig. 3.1 using oligonucleotides as presented in schematic panel in Fig. 3.1. The average of at least two independent experiments is shown. Error bars in the graph represent standard deviation (S.D.) while asterisks denote the calculated p-value ** $p<0.01$, * $p<0.05$

Next, we wanted to see if Rho expression has any effect on Sub2 recruitment upon RNase treatment, a factor previously shown to be RNA-dependent (Abruzzi et al., 2004; Meinel et al., 2013). The experiment on tagged Sub2 strain was performed in the same way as for Tho2. We found RNA dependence in RNase treated strains without Rho expression to be in accordance with previously known results (Figure 3.10A). Samples with active Rho

expression system show the same decrease in recruitment level upon RNase treatment as established for samples without Rho presence (compare Figures 3.10A and B). Hence, the effect of RNase treatment is the same whether Rho is expressed or not, meaning that the RNase sensitivity of Sub2 is independent from Rho action (Figure 3.10C).

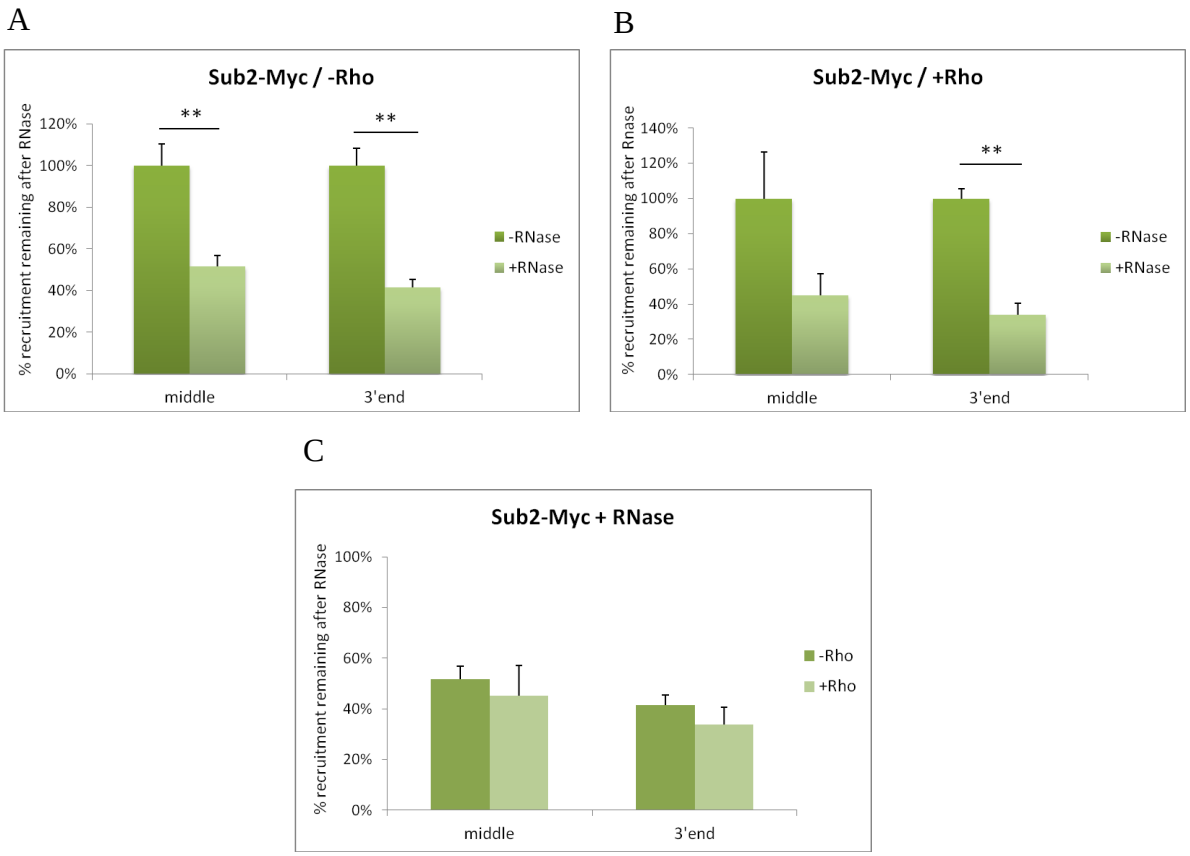


Figure 3.10: *Rho action shows no significant influence on RNase sensitivity of Sub2 recruitment to transcription site*. (A) ChIP results for Myc-tagged Sub2 strain harboring an empty vector pCM185, with and without RNase treatment. Results presented as in Fig 3.9A. (B) ChIP results for Myc-tagged Sub2 strain harboring Rho expression vector pCM185-Rho-NLS, with and without RNase treatment. Results presented as in Fig. 3.9A. (C) Comparison of percentages of Sub2 recruitment after RNase treatment relative to the untreated samples, without (*-Rho*) and with *(+Rho*) Rho expression. ChIP experiment was performed as in Fig. 3.1 using oligonucleotides as presented in schematic panel in Fig. 3.1. The average of at least two independent experiments is shown. Error bars in the graph represent standard deviation (S.D.) while asterisks denote the calculated p-value ** $p<0.01$, * $p<0.05$

Rho action disturbs the THO complex (removing Mft1) which leads to RNase sensitivity of THO complex, not observed in the absence of Rho. However, RNase sensitivity of Sub2 is not influenced by Rho action, which can be explained by the proposed Sub2 transfer onto the transcript after its recruitment as a part of TREX complex.

3.2.2 THO-Sub2 complex and the deletion of Mft1

Les résultats des expériences de ChIP obtenus pour les membres du complexe THO-Sub2, notamment les profils différents de recrutement de Mft1 dans le système d'expression Rho comparativement aux autres protéines du complexe, nous ont amené à visualiser le comportement des membres de THO-Sub2 dans une souche mft1Δ. *Pour toutes les protéines THO-Sub2 testées, l'intensité du signal ChIP dans la souche* mft1Δ *est grandement réduite en comparaison avec l'intensité observée dans la souche sauvage, mais nous avons trouvé une diminution du niveau de protéines cellulaires seulement pour Tho2. L'action de Rho et la délétion de Mft1 mènent toutes deux à la rétention du transcrit dans les corps nucléaires et, en combinant ces deux systèmes expérimentaux, nous observons une récupération partielle de la dégradation du transcrit accompagnée par le recrutement de Sub2 au transcrit à un niveau comparable à celui dans la souche sauvage.*

3.2.2.1 THO-Sub2 in strains with *mft1Δ* background

ChIP results obtained for THO-Sub2 members, namely the different recruitment pattern of Mft1 in Rho expression system compared to the other observed proteins, led us to visualize the behavior of THO-Sub2 members in a strain with *mft1Δ* background. Previous studies have shown that deletion of one member of the THO complex influences the stability of the whole complex, and by consequence leads to a low ChIP recruitment profile of other members of the complex, as well as Sub2 (shown in *hpr1Δ* strain) (Huertas et al., 2006; Pfeiffer et al., 2013). Therefore, we sought to show the expected decrease in ChIP recruitment profile in *mft1Δ* background for THO-Sub2 complex members of interest (Tho2, Hpr1 and Sub2).

ChIP was performed by the same protocol as before, on strains with Myc-tagged proteins of interest and with or without a deletion of *MFT1* (Figure 3.11). The observed results are as expected. For all tested THO-Sub2 proteins the level of ChIP signal in the *mft1Δ* strain is vastly reduced in comparison with the level in the wt strain.

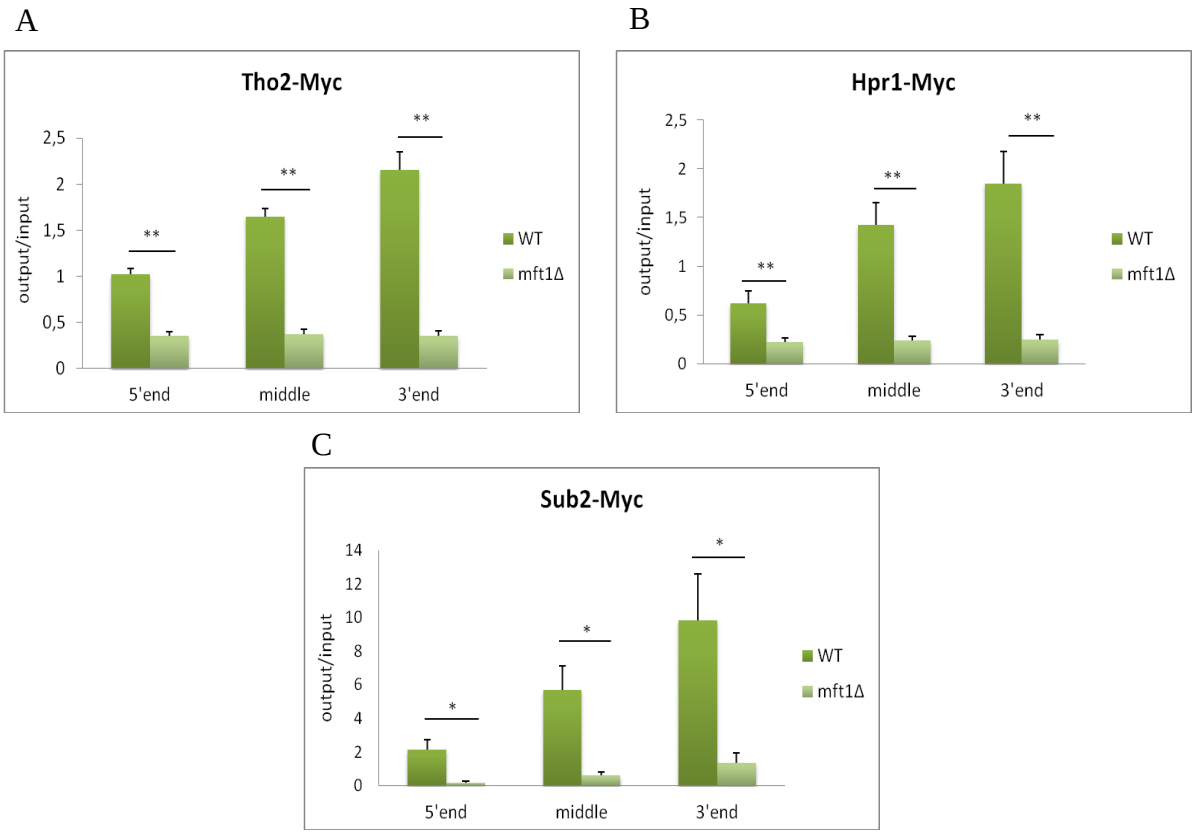


Figure 3.11: *Recruitment of THO-Sub2 members in mft1Δ yeast strains is significantly decreased*. Quantification of the fold enrichment for PMA1 DNA in BMA41 strain with a Myc tag on (A) Tho2, (B) Hpr1 and (C) Sub2 proteins coupled with a wt or mft1Δ background. ChIP experiment was performed as in Fig. 3.1 using oligonucleotides as presented in schematic panel in Fig. 3.1. The average of at least four independent experiments is shown. Error bars in the graph represent standard deviation (S.D.) while asterisks denote the calculated p-value ** $p<0.01$, * $p<0.05$.

To determine the stability of THO-Sub2 protein subunits in *mft1Δ* strains we performed Western blot on the same strains we used for ChIP. As shown in Figure 3.12, from the proteins tested only Tho2 stability seems to be affected by the *MFT1* deletion, while other proteins are present at the same level in wt and *mft1Δ*. This is in accordance with previous results (Huertas et al., 2006; Pfeiffer et al., 2013) where it was also shown that THO2 transcript levels were similar in wt and a strain lacking one THO subunit, meaning that the decreased level of Tho2 protein results from a posttranscriptional effect.

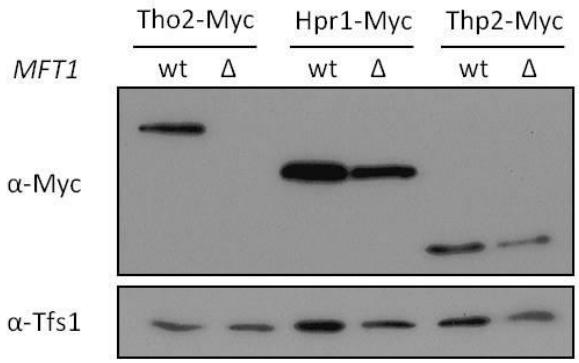


Figure 3.12: *Protein level of Tho2 is decreased in mft1Δ strain, but not for other members of THO complex.* Western blot analyses of whole protein extracts isolated from strains harboring Myc-tagged THO subunits in wt or mft1Δ background and detected with anti-Myc antibodies (*α-Myc*). As in Fig. 3.4, including the detection of the loading control protein, Tfs1.

3.2.2.2 Comparison between Rho expression system and *mft1Δ* background

Rho expression and *MFT1* deletion are two completely different systems with different levels of influence on transcript steady state levels within yeast. However, a comparison between these two systems for their impact on the THO complex and PMA1 transcript might lead to some conclusions about THO function and Rho influence on transcription and/or QC.

First noticeable difference between these two systems is the stability of THO protein subunits and the complex itself. The results in the previous section show that the *MFT1* deletion leads to degradation of another THO complex member, Tho2 (section 3.2.4.). The lack of only one protein subunit already affects the stability of the whole complex whose presence can be detected only in trace quantities (Huertas et al., 2006). This impact on THO complex formation is substantiated by ChIP results showing significantly decreased recruitment of THO complex members to chromatin in *mft1Δ* background (Pfeiffer et al., 2013; Figure 3.11). On the contrary, in Rho expression system THO complex members are not degraded nor is their presence in the cell altered and according to their ChIP recruitment profiles it is not hard to presume that besides Mft1, other members continue to persist as a

complex (section 3.2.1; section 3.2.2). This observation suggests a crucial role of Mft1 in the THO complex, enabling its proper function in mRNP biogenesis.

THO-Sub2 mutants show retention of aberrant transcripts at the transcription site foci visualized by FISH assay using sequence specific probes (Libri et al., 2002; Jensen et al., 2004). We used the same technique to compare the impact of *MFT1* deletion and Rho expression on transcript nuclear sequestration. We found similar levels of transcript retention in nuclear dots in both experimental systems and the expected lack of nuclear dots in –Rho strains, which portray a wt phenotype (Figure 3.13).

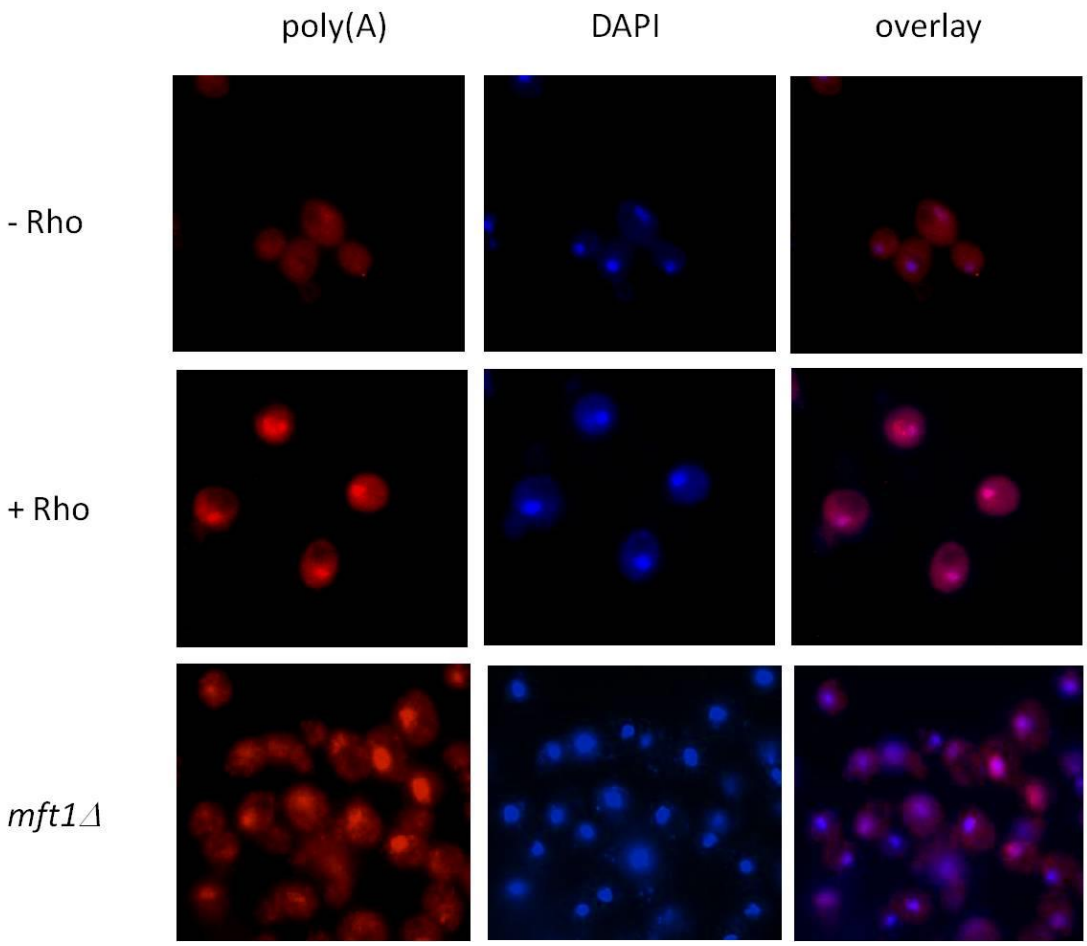


Figure 3.13: *Nuclear mRNA retention is comparable between mft1Δ strain and wt strain with Rho expression system*. BMA41 strain harboring Rho expression plasmid (pCM185-Rho-NLS) was grown under Rho repressing or inducing conditions at 25˚C, while mft1Δ strain was shifted to 37˚C for 15 min before FISH protocol. The mRNA was detected using a Cy3-labeled 40-mer d(T) oligonucleotide as a probe directed against the poly(A) tails. Nuclear DNA was stained with DAPI.

After demonstrating a similar effect on transcript retention in yeast cells, we decided to compare the impact of these two systems on transcript degradation, namely PMA1. The effect of Rho expression on PMA1 transcript is presented in section 3.2.1 (Figure 3.5B). In samples obtained from strains with Rho expression we observe a large decrease in PMA1 abundance which is due to QC degradation (Honorine et al., 2011; Mosrin-Huaman et al., 2009). In THO-Sub2 mutants there is a different degree of transcript degradation which varies between different transcripts (Libri et al., 2002; Zenklusen et al., 2002; Jensen et al., 2004b; Garcia-Rubio et al., 2008). The effect on PMA1 transcript has previously only been tested in *hpr1Δ* strain where the level of PMA1 transcript was not affected in strains grown at 25˚C (Zenklusen et al., 2002).

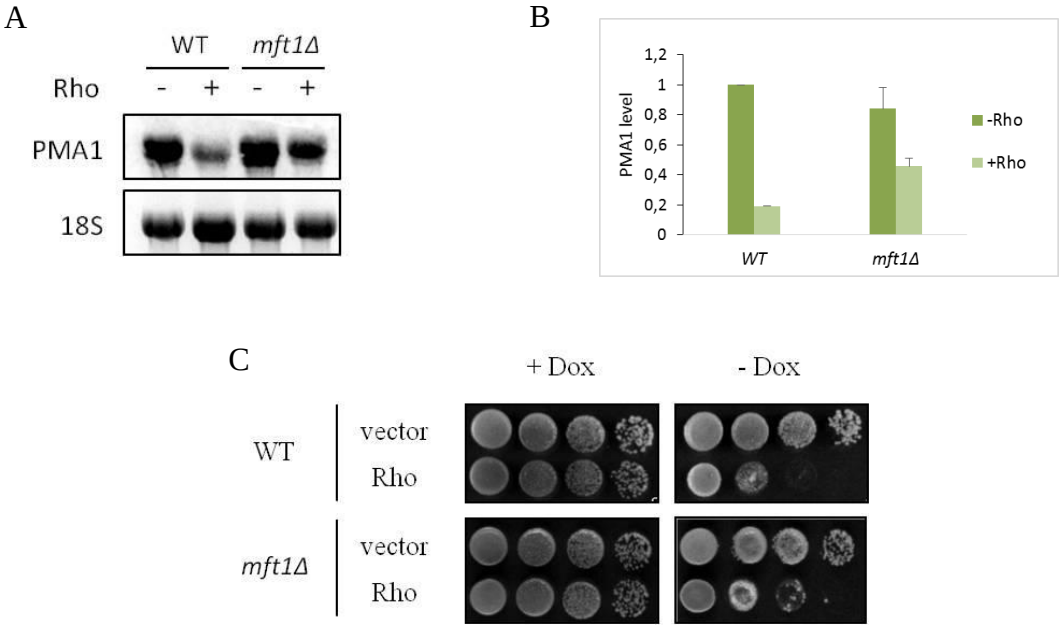


Figure 3.14: *MFT1 deletion in Rho expression system partially reliefs PMA1 transcript degradation, but not the growth defect*. (A) Northern blot assay on total RNAs extracted from BMA41 wt or *mft1Δ* strain transformed with the Rho expression plasmid and grown in Rho repressing or inducing conditions, as in Fig. 3.2B. (B) Quantitative RT-PCR analysis performed on total RNA (1 µg) samples from strains as in (A). Histogram shows PMA1 abundance relative to 18S rRNA (amplicon 197). PMA1 signal for BMA41 wt strain in absence of Rho-expression is set to 1 as a reference value. The average of three independent experiments is shown, with error bars representing S.D. (C) Serial dilution test performed as described in Fig. 3.2A on BMA41 wt or *mft1Δ* strain transformed either with a plasmid without Rho expression system (pCM185) or the plasmid expressing Rho (pCM185-Rho-NLS).

We performed Northern blot assay using PMA1 specific probes to detect and compare PMA1 transcript levels in wt strain with Rho expression and in strain with *mft1Δ* background. We also constructed an *mft1Δ* strain coupled with Rho expression and performed the same assay. As expected from the known data, we found a large decrease in PMA1 level in strain with Rho expression while the level in *mft1Δ* strain was the same as in the wt strain without Rho (Figure 3.14A). Interestingly, for the *mft1Δ* strain under Rho expression the results show a partial rescue of the PMA1 transcript degradation compared to the wt strain with Rho expression. RT-PCR quantifications of the PMA1 mRNA are consistent with the Northern blot results, by detecting 20-30% higher level of transcript under Rho expression in *mft1Δ* strain, relative to the wt (Figure 3.14B). Moreover, the serial dilution test of *mft1Δ* strain with Rho expression shows a slight relief of growth defect, which correlates with the observed transcript rescue (Figure 3.14B). These results suggest that Rho action, in the absence of a full THO complex, induces an alternative pathway in mRNP biogenesis which enables a partial transcript and growth rescue.

We obtained additional input regarding the observed partial transcript rescue in *mft1Δ* strain with Rho expression by performing ChIP assay on tagged Sub2 in *mft1Δ* strains with and without Rho expression. Results presented in section 3.2.2.1 show that Sub2 recruitment to PMA1 chromatin is hardly detected in the strain lacking MFT1 (*mft1Δ*). The same result is attained in Sub2-Myc *mft1Δ* strain without Rho while in *mft1Δ* with expressed Rho the ChIP signal again displays high chromatin recruitment, comparable to the one in the wt strain (Figure 3.15, compare with Figure 3.8D). It is easy to make an assumption that this restored Sub2 recruitment could be the reason for the observed partial PMA1 transcript rescue and growth defect relief in *mft1Δ* under Rho expression (see Discussion).

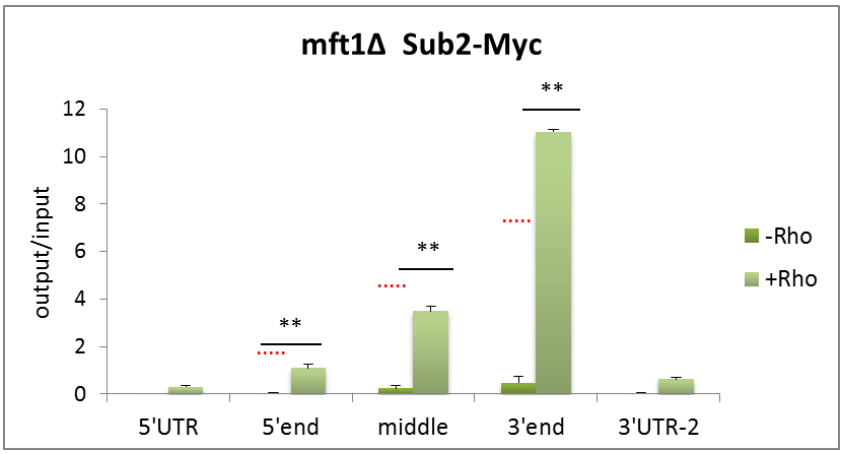


Figure 3.15: *Rho action in mft1Δ strain reconstitutes Sub2 recruitment to the transcription site*. Quantification of the fold enrichment for PMA1 DNA in immunoprecipitates from mft1Δ strains harboring Myc tagged Sub2 and the Rho expression vector (pCM185-Rho-NLS). Yeast strains were grown either in repressing (-*Rho*) or inducing (+*Rho*) conditions. ChIP experiment was performed as in Fig. 3.1 using oligonucleotides as presented in schematic panel in Fig. 3.1. The average of three independent experiments is shown. Error bars in the graph represent standard deviation (S.D.) while asterisks denote the calculated p-value ** $p<0.01$, * $p<0.05$ Red dots depict the appropriate signal levels from Fig. 3.8D for Sub2-Myc/-Rho for easier comparison.

4 DISCUSSION

In previous publications the bacterial termination factor Rho was proven to be a global disturber of gene expression while expressed in yeast (Mosrin-Huaman et al., 2009; Honorine et al., 2011; Stuparevic et al., 2013). However, the nature of this disruption has yet to be fully elucidated and the work presented in this manuscript contributes to the aim of better understanding this phenomenon.

4.1 Rrp6 is the main nuclease degrading the Rho-induced aberrant transcripts

Rho factor is a helicase/translocase which loads onto exposed C-rich sequences of the nascent transcript and tracks towards the transcribing RNA polymerase in an ATP dependent manner. *In vitro* studies demonstrated that Rho is a powerful molecular motor capable of breaking RNA-protein connections (Lang et al., 1998; Schwartz et al., 2007a). In yeast, conditionally expressed Rho factor leads to dose-dependent growth defect phenotype which is presumed to be the result of the aforementioned functional characteristics of Rho protein (Mosrin-Huaman et al., 2009). In the proposed model, the loading and translocation of Rho factor on the nascent transcript competes with the deposition and function of processing and/or packaging factors in the mRNP biogenesis pathway. This deprivation of key biogenesis factors leads to the production of aberrant transcripts consequently recognized and degraded by the nuclear RNA degradation machinery. The latter was substantiated by growth defect relief in strains with mutations of the nuclear exosome component Rrp6 (Mosrin-Huaman et al., 2009). The same study reveals that *RRP6* deletion rescues the Rho-induced degradation of transcripts. Hence, we sought to determine the extent of Rrp6 involvement in this degradation process in comparison with other exosome components.

The eukaryotic exosome complex is a versatile processing and degradation machinery, operating in transcript degradation as a part of their regular turnover or as a final step in different quality control processes. It is composed of a conserved, catalytically inactive core and two associated catalytically active components, Dis3 and Rrp6. Dis3 is an essential active subunit of the exosome, ubiquitously present in the cytoplasm and in the nucleus. It has both endonuclease and 3’ to 5’ exonuclease activities involved in many transcript processing and degradation steps. Rrp6 is a non-essential nuclear component of the exosome with a 3’ to 5’ exonuclease activity. It functions in different processing and degradation activities, and its

presence enhances the activity of Dis3. However, the extent to which Rrp6 activity is dependent on the presence of the core exosome is not known.

To complement our earlier results showing Rrp6 involvement in degradation of Rho-induced aberrant transcripts, we constructed a yeast strain with an exonuclease deficient *rrp6-Y361A* mutant and coupled it with Rho expression system. This catalytically inactive mutant exhibits the same growth phenotype suppression as the full *RRP6* deletion. Likewise, Northern blot and RT-PCR quantification analyses in *rrp6-Y361A* under Rho expression show similar PMA1 transcript rescue than the one observed in *rrp6Δ*. Together with the result that Rrp6 recruitment to the transcription site is not affected by the *Y361A* mutation, it is clear that the exonuclease function of Rrp6 plays a major role in degradation of Rho-induced aberrant transcripts. This conclusion is substantiated by the results of the same experimental analyses performed on constructed *dis3* mutant strains, lacking either the endonuclease or exonuclease activity. Indeed, there is no growth rescue in the presence of Rho for either of the two *dis3* strains and only a slight (10%) rescue of PMA1 transcript is observed for the endo^{-} strain. Hence, Rrp6 is the main catalytic component of the exosome responsible for the degradation of aberrant transcripts in Rho expression system, with a probable minor involvement of Dis3. Still, Dis3 and the exosome may have a more significant role in degrading other transcripts or as a part of different quality control systems. Indeed, recent transcriptome-wide analyses in budding yeast showed that Rrp6 and Dis3 have both specific and overlapping targets, with Dis3 being even more represented in the coding transcriptome, especially in degrading intron-containing pre-mRNAs (Gudipati et al., 2012; Schneider et al., 2012). Due to the moderate recruitment signals for Dis3 and the core exosome subunits we obtained in our ChIP experiments, we also cannot exclude the possibility of Rrp6 dependence on the presence of the exosome to deliver the substrate RNA through its central channel (Wasmuth et al., 2014). However, these modest recruitment levels of Dis3 and exosome components and the fact that they are not Rho-dependent, could also be explained in the light of recently proposed model of a constitutive, competitive QC described in section 1.2.3.3. Next, we turned to the exploration of the nature of Rho-induced transcript defect which leads to transcript retention and degradation by Rrp6.

4.2 Rho action reveals a surprising behavior of Mft1 and a "hidden" RNA dependence of the THO complex

TREX complex is a pivotal factor in coupling and managing different steps of mRNP biogenesis. It consists of THO subcomplex (Tho2, Hpr1, Mft1, Thp2 and Tex1) and export factors Sub2 and Yra1. Together, they recruit and interact with numerous factors involved in transcription, splicing, 3'end processing and export. Indeed, this network of interactions enables TREX to perform its main role in promoting high-speed, efficient transcription, thus securing optimal kinetics for mRNP biogenesis. Mutant strains bearing null mutations of members of TREX complex have also been extensively used in most research of yeast nuclear quality control mechanisms. Given the importance of TREX complex in nuclear steps of gene expression and its established use in the study of QC surveillance, we found its members to be an excellent choice as targets for our study of Rho interplay with mRNP biogenesis factors.

Chromatin immunoprecipitation is a powerful technique to conduct research on protein recruitment to chromatin. It is also useful in the study of recruitment profile of different factors which act during transcription. In the initial work done in our laboratory with Rho factor as a tool, we found that the simultaneous use of Rho expression and ChIP techniques shows no secondary effects which would affect the results of protein recruitment profiles (Honorine et al., 2011). In the research presented in this manuscript we used ChIP technique to observe the influence of Rho presence on the recruitment profile of several mRNP biogenesis factors, namely THO-Sub2 members. We constructed Myc-tagged target proteins of interest (Tho2, Hpr1, Mft1 and Sub2) to enable the use of specific anti-Myc antibodies for protein immunoprecipitation. Since the presence of the additional tag sequence adjacent to the protein of interest might disturb normal structure, function and interactions of tagged proteins, we verified that Rho effect on yeast phenotype and PMA1 transcript is not altered by addition of a tag to specific protein factors of interest. A serial dilution test confirmed that the yeast growth defect phenotype under Rho expression is not affected by the presence of a Myc tag on any of the proteins of interest. Northern blot analysis also confirmed that the Myc tag does not influence normal, steady state PMA1 level in wt, nor its degradation in the strain with Rho expression. We also investigated the possibility that the presence of Rho protein, due to its influence on transcript degradation, could alter expression of the target

proteins. Western blot analysis of Myc-tagged proteins of interest in the presence or absence of Rho factor showed no variation in the level of expressed proteins.

Recent results show that THO complex is recruited to the transcription site through interactions with the Ser2/Ser5 phosphorylated CTD of Pol II (Meinel et al., 2013). Indeed, the ChIP recruitment profiles of THO-Sub2 members show an increase in recruitment from 5' to 3'end of the transcribed gene, which resembles to the phosphorylation pattern of CTD Ser2 mark (Abruzzi et al., 2004; Ahn et al., 2004; Kim et al., 2004). This recruitment profile is unique among the transcription factors and the explanation for this behavior is still inconclusive. We set up ChIP experiment using the aforementioned verified THO-Sub2 tagged strains in which we inserted either an empty vector or a vector carrying the Rho expression system. ChIP results obtained by quantitative PCR for tagged strains carrying an empty vector confirm previous results and known behavior of THO-Sub2 recruitment profiles (Zenklusen et al., 2002; Abruzzi et al., 2004). All tested proteins show an increase in recruitment from 5' to 3' end and a lack of recruitment in the UTR surrounding the *PMA1* gene. According to the previously described model we envisaged, we expected the THO-Sub2 recruitment profile will be decreased as a result of Rho action disrupting the deposition or removing the complex from the transcript. However, in strains with Rho expression we observed different behaviors between members of the THO-Sub2 complex. The only target protein which behaved according to our expectations was Mft1, with a two and a half times decrease in mean ChIP signal at the 5' end, to more than 4 times decrease at the 3' end. Other observed THO-Sub2 members (Tho2, Hpr1 and Sub2) show a trend opposite from the one displayed by Mft1. They show a significant increase in ChIP signal, with almost a two times increase at the 3' end for Sub2 and a three and a half times increase at the 3' end for Tho2. The increase in ChIP signal could arise from an increase in recruitment to the Rho perturbed transcript, but could also result from better accessibility of tag epitopes to immunoprecipitation with antibodies (Weinmann, 2004; Xiao, 2006). This epitope exposure could be the consequence of Rho induced perturbance of THO-Sub2 complex and/or the mRNP structure of the transcript in general, due to Rho ability to disrupt RNA-protein complexes present on its path and to melt nucleic acid base pairs (Walmacq et al., 2006; Schwartz et al., 2007a).

Mft1 is a poorly characterized protein, initially reported to be involved in mitochondrial protein targeting due to a reduced level of mitochondrial accumulation of *lacZ*

fusion protein in *mft1* mutant (Beilharz et al., 1997). However, further research showed Mft1 is actually a subunit of THO complex whose hallmark phenotype is the inability to transcribe lacZ if any of the subunits is deleted, which explains the initial result (Chavez et al., 2000). The null mutant of *mft1* shows the same transcription, hyper-recombination and temperature sensitivity phenotype, although to a lesser extent than in *tho2* and *hpr1* null mutants (Libri et al., 2002; Garcia-Rubio et al., 2008). Evidence suggest that the N-terminal domain of Mft1 is required for interaction with other members of the THO complex and that the C-terminal domain is an acidic disordered region which may act as an interaction platform required for protein-protein interactions during mRNP biogenesis (Chavez et al., 2000; Poulsen et al., 2014). Aside from this information not much is known about Mft1 structure, role or interactions, which makes it hard to explain the difference in behavior from other THO-Sub2 members in our ChIP experiment with Rho expression system.

In order to get more information about the possible function and characteristics of Mft1 as a member of THO complex and in the process of QC, we decided to make a deletion strain for this protein. We combined this deletion with a Myc tag on other members of THO-Sub2 to see how this deletion influences the ChIP recruitment profile of other THO-Sub2 members at *PMA1* gene locus. As previously shown for some members of THO-Sub2 (Huertas et al., 2006; Pfeiffer et al., 2013), we found the recruitment of our target factors (Tho2, Hpr1 and Sub2) almost completely abolished in *mft1Δ* strain. We explain these results by the fact that in a single deletion mutant of THO complex the stability of the whole complex is greatly reduced, as demonstrated by Huertas et al. (2006). The same study also suggested that THO complex protein subunits may only be stable in a form of a complex and degraded if complex formation is disturbed by the lack of one of its members. We performed Western blot on tagged members of THO complex in *mft1Δ* background strain and found that only the protein level of Tho2 has been reduced while levels of other members (Hpr1 and Thp2) were not affected. This decrease in Tho2 is in accordance with previous results where it was also shown that THO2 transcript level is not impacted, therefore this is the result of a posttranscriptional effect (Huertas et al., 2006). Next, we decided to combine *mft1* deletion with Rho expression system. It has been shown before that the PMA1 transcript level is not reduced in *hpr1Δ* strain grown at 25˚C and we obtained the same results for *mft1Δ* strain without Rho expression. However, contrary to the strongly reduced transcript level in wt strain under Rho expression, in *mft1Δ* strain with Rho expression we observed a partial rescue

of the transcript. Furthermore, this transcript rescue was accompanied by a growth defect relief, as revealed by the serial dilution growth assay. In the time frame of this project we managed to construct and perform ChIP assay on only one *mft1Δ* strain with a tagged member of THO-Sub2 complex combined with the Rho expression system. The tagged protein of interest was Sub2 which demonstrated an increased ChIP signal in wt strain with Rho expression and a lack of recruitment in *mft1Δ* strain (see sections 3.2.2 and 3.2.4). Interestingly, in *mft1Δ* strain with Rho expression system, Sub2 once again displays high ChIP signal, comparable to the one in wt strain without Rho. We suggest that this increase in recruitment might be due to a Rho-induced stimulation of an alternative Sub2 recruitment pathway in *mft1Δ,* which may provide an alternative pathway for mRNP biogenesis in yeast THO mutants (Fan et al., 2001; Zenklusen et al., 2002; Jimeno et al., 2006; Yu et al., 2012). It would be interesting to combine Rho expression system with the *hpr1* point mutation presented in Huertas et al., (2006), which reduces Sub2 recruitment to transcription site without affecting the stability of THO complex. This experiment would reveal if this proposed alternative recruitment pathway of Sub2 can function even in the presence of THO complex or if the lack of THO is necessary for its activation. Given the fact that Sub2 overexpression suppresses the Rho induced growth phenotype and partially rescues PMA1 transcript from degradation (Mosrin-Huaman et al., 2009), we also suggest that the increased recruitment of Sub2 in *mft1Δ* strain under Rho expression is the reason for the observed partial growth defect relief and PMA1 transcript rescue in the same strain.

Table 4.1: Comparison of phenotypes between *Δmft1* mutant, Rho expression and the combination of the two systems.

	Δmft1	Rho expression		
THO complex stability	- perturbed, Tho2 degraded	- maintained without Mft1?	(Huertas et al., 2006; This study)	
Transcript nuclear retention	- nuclear dots (at 37°)	- nuclear dots	***Δmft1* + Rho expression**	(Libri et al., 2002; Jensen et al., 2004; This study)
PMA1 transcript degradation	- not degraded (HSP104 3' end degraded at 37°)	- degraded	- partialy degraded/rescued	(This study)
Growth defect	- no growth defect (at 25°C)	- sever growth defect	- growth defect partialy relieved	(Libri et al., 2002; This study)
Sub2 chromatin recruitment	- vastly reduced	- increased	- increased	(This study)
Combined with Rrp6 deletion	- growth defect aggravated with higher temperature	- growth defect relieved	/	(Libri et al., 2002; Mosrin-Huaman et al., 2009; This study)
Combined with a reduced rate of transcription	- rescue of the hyper-recombination, retention and degradation phenotype	- aggravated growth defect phenotype	/	(Jensen et al., 2004; Jimeno et al., 2008; This study)

One of the shortcomings of ChIP technique used in research of protein factors recruited to transcription site is that it does not give the information of the direct interaction site in recruitment. It does not reveal if a factor is recruited to chromatin, transcript or is it in connection only to other proteins. During formaldehyde crosslinking all nucleic acid-protein and protein-protein connections get fixed. This means that theoretically for each inspected chromosomal locus we can get a ChIP signal for each protein present from the transcriptional machinery to the proteins of the CBC. To obtain information on interaction locus for a specific protein, other techniques are combined with ChIP analysis (e.g. RNase treatment, ribozyme cleavage, mutations) and complemented with results from other experimental procedures (e.g. affinity purification and techniques similar to ChIP, like cross-linking and immunoprecipitation (CLIP) and UV cross-linking and analysis of cDNAs (CRAC) used in studies of RNA bound proteins and in generating genome-wide transcriptome maps). ChIP results for TREX members show an intriguing 5' to 3' increase in recruitment along the observed gene, demonstrated for no other transcription factor (Abruzzi et al., 2004; Gomez-Gonzalez et al., 2011; Meinel et al., 2013; Katahira, 2015). *In vitro* studies determined THO complex binding to both DNA and RNA substrates (Jimeno et al., 2002; Pena et al., 2012). Hence, a hypothesis was made that the 5’ to 3’ increase in recruitment of TREX members

reflects its binding along the nascent transcript. However, ChIP experiment combined with RNase treatment before the immunoprecipitation step showed no significant difference in recruitment of THO members compared to the non-RNase treated samples (Abruzzi et al., 2004; Pena et al., 2012). Nonetheless, the same study demonstrated RNA dependence in recruitment of Sub2. The lack of THO dependence on RNA shown by these results suggests that THO members are not recruited along the nascent transcript but rather to the chromatin and the transcription machinery. Additional input in this matter was provided by affinity-purification and transcriptome mapping of yeast mRNPs. Interestingly, these techniques showed that THO members are undoubtedly bound to the mRNA, even more so, with a higher occupancy at the 5' end of the transcript (Batisse et al., 2009; Tuck and Tollervey, 2013; Baejen et al., 2014; Bretes et al., 2014). With all these results combined the matter of THO interactions at the site of transcription still remains vague.

In our ChIP experiments on THO-Sub2 members in systems with and without Rho expression, we decided to subject our samples to RNase treatment before immunoprecipitation step, as was previously done by other research groups. PCR quantification results of ChIP for TREX member Sub2 with a Myc tag and without Rho expression confirmed what has previously been published by other research groups. Sub2 recruitment decreased upon RNase treatment, which confirms previously suggested RNA dependence in recruitment of this factor. In contrast, Rnase treatment did not modify the Sub2 recruitment under Rho expressing conditions. We performed the same experiment on Tho2-Myc tagged strains. Again, our results without Rho expression were in accordance with previous results. We found no significant difference in recruitment of Tho2 with and without RNase treatment. However, when we combined RNase treatment and Rho expression system we obtained intriguing results. In samples with Rho expression and treated with RNase we observed a very significant (70%) decrease in Tho2 recruitment to the transcription site, in comparison with the recruitment level in samples with Rho expression but not treated with RNase. These results clearly demonstrate that Rho action perturbs THO complex in such a way that it exposes the recruitment of THO complex to nascent transcript which cannot be perceived by ChIP experiment performed on wt strains. What is the reason behind this? There is the possibility of other additional THO complex interactions aside from RNA binding, which get disrupted by Rho action. Meinel and Sträßer, (2015) propose bifunctional binding of TREX complex to Pol II CTD and RNA to ensure spatial proximity of the nascent mRNA

to mRNP binding proteins recruited to the CTD. This is in accordance with our hypothesis since TREX interaction with the CTD would ensure TREX direct crosslinking to the transcription machinery in ChIP experiment, which would mask the RNA dependence of TREX members upon RNase treatment. The observed dependence of Sub2 on RNA binding can be explained by the Sub2 recruitment by the THO complex and it subsequent transfer onto the mRNA (Abruzzi et al., 2004). This conclusion is corroborated by the fact that although Sub2 presence at the transcription site is RNA dependent in ChIP experiment, its interaction with THO complex does not show RNase sensitivity (Strasser et al., 2002). Mft1 is a factor potentially involved in bridging the THO complex RNA/CTD connection. According to our results, we can envisage that Rho action disturbs and dissociates Mft1 from the THO complex, thus removing the connection with the CTD, while other members of THO complex stay bound to the RNA (Figure 4.1 A). However, in this model it is hard to argue why would Rho action disturb THO interaction with the CTD but would not dissociate this complex from the mRNA along which the Rho factor tracks. Another matter is the length of the CTD which can vary from 100 Å (in its hypophosphorylated state) to 700 Å (fully extended) (Meinhart and Cramer, 2004; Meinel et al., 2013; Meinel and Sträßer, 2015). This length corresponds to ~350 nt to ~2500 nt long extended mRNA, respectively. Length of the CTD probably changes with its different phosphorylation status, but it most probably does not exist in its fully extended form. The question is: would the CTD be able to span the whole length of a transcript (average 1436 nucleotides) which would be necessary to secure THO binding at the 5' end, due to the fact that THO recruitment was found to be most important in transcription of long transcripts and that transcriptome mapping shows highest occupancy of THO at the 5' end of transcripts (Gomez-Gonzalez et al., 2011; Meinel et al., 2013; Tuck and Tollervey, 2013; Baejen et al., 2014). Given that the actual length of the CTD during transcription, as well as the level to which the nascent mRNA is compacted are not known, it is hard to argue for or against the proposed model of TREX recruitment and simultaneous binding of the CTD and RNA as the sole reason for concealed RNA dependence of THO.

We can propose another model which could circumvent the uncertainties regarding the CTD/mRNP length and can also provide an explanation to how could most of TREX members stay bound to the transcript following Rho action. This model has the same principal of recruitment and binding to the CTD as the first model. However, we suggest that in addition, THO can make multimers which would secure mRNA compactness and vicinity to

the CTD even when the length of the transcript exceedes the length of the CTD (Figure 4.1 B). This idea is affirmed by the observation that a four-subunit THO complex (Tho2, Hpr1, Mft1 and Thp2) is affinity purified as a ~1400 kDa complex, which is much bigger than the predicted ~350 kDa for this complex (Chavez et al., 2000). We also suggest that Mft1, in addition to its supposed interaction with the CTD, might be responsible for this multimerization of THO complex due to the fact that it is the only one removed from the transcription site by Rho action and, as previously mentioned, it has a flexible C-terminal domain which could serve in protein-protein interactions (Poulsen et al., 2014). Since this model predicts that TREX is multimerized and tightly compacted with mRNA, it would also explain why we do not observe removal of the complex by Rho action, but quite the opposite, we observe a significant increase in ChIP signal (except Mft1). This would be the result of a better accessibility of epitopes recognized by the immunoprecipitation antibodies, following Rho perturbation of THO-Sub2 complex. We also suggest that the compactness of the mRNP allows the THO complex to again quickly bind to the mRNA after its displacement by Rho action. However, this would also lead to removal of Mft1 subunit and disruption of other lower affinity interactions (e.g. with the CTD) which would allow better accessibility of the complex to immunoprecipitation antibodies. Nevertheless, the proposed hypothesis and described models are highly speculative and a lot more research is necessary to complete the picture of TREX binding to transcription site and Rho interplay with mRNP biogenesis factors.

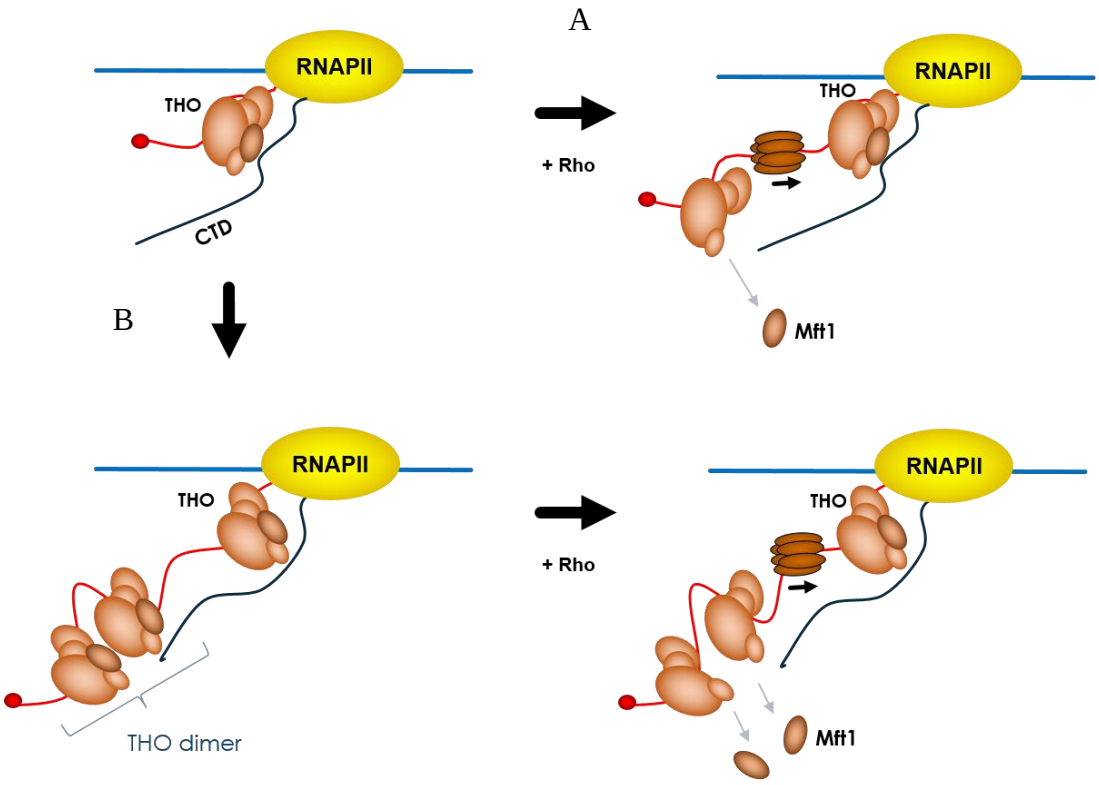


Figure 4.1: *Models depicting the proposed action of Rho factor in perturbing THO complex stability.* (A) Rho action displaces Mft1 from the THO complex and disrupts the physical connection between THO complex and the CTD. (B) In addition to disrupting THO binding to the CTD, Rho also brakes up multimers of THO complex formed after the length of the transcript surpasses the length of the CTD.

In conclusion, the results presented in this manuscript give further insight into the nuclear quality control mechanism provoked by Rho action, showing that Rrp6 is the main degradation factor involved in this process. Involvement of the core exosome and its cofactor Dis3 in degradation of Rho-induced aberrant transcripts was shown to be only minor, although their overall contribution to the process, maybe by promoting Rrp6 function, cannot be discarded. In the second part of this work, we showed that Rho action indeed does disturb the mRNP biogenesis, by perturbing the stability of a key mRNP biogenesis complex – TREX. Rho action causes the removal of Mft1 from the THO complex and the transcription site, while other members of THO-Sub2 complex become more accessible to immunoprecipitation by antibodies used in our ChIP experiment. Interestingly, Rho action

makes the remaining members of THO-Sub2 complex sensitive to RNase treatment, thus revealing their dependence and connection to the nascent transcript, expected to be the cause for THO-Sub2 unique increasing ChIP recruitment towards the 3' end of genes. To explain this phenomenon we propose a model where THO-Sub2 complex bridges the interaction between the nascent RNA and the CTD, thus keeping them in proximity necessary for efficient mRNP biogenesis, while THO-Sub2 also forms multimers, compacting the mRNP which partially masks the ChIP signal obtained for THO-Sub2 members in wt strains and enables their reattachment to the nascent transcript following Rho action. As the third part of our research, we compared and combined Rho expression system and the deletion of *MFT1*. In *mft1Δ* strain with integrated and activated Rho expression system, we observed a partial rescue of growth defect and PMA1 transcript degradation, accompanied by the restoration of Sub2 transcription site recruitment, as revealed by ChIP. Since Sub2 overexpression has been shown to suppress THO single null mutant phenotype, we suggest that Rho action in *mft1Δ* strain might activate an alternative Sub2 recruitment pathway which could bring upon the partial transcript escape from quality control degradation and consequently lead to a slight rescue of the growth defect observed for *mft1Δ* strain without the presence of Rho factor. Results presented in this study open a lot of new questions and possible hypothesis which can be developed for future projects. Regarding nuclear quality control and degradation by Rrp6 we already revealed involvement of the TRAMP complexes, however, the precise mechanism by which the TRAMP complexes stimulate Rrp6p activity requires further work to be completely elucidated. In the subject of THO complex we can envisage to confirm by additional experimental techniques the bifunctional binding at the transcription site and/or the involvement of Mft1 in this process and possibly in nuclear surveillance. An interesting is also raised at the possibility of an alternative Sub2 recruitment pathway and could be envisaged as an independent project. In summary, our results and conclusions are just an introduction to possible studies necessary to unravel this complex processes, functions and interactions comprising the complete mRNP biogenesis.

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Mateja REMENARIC HAJAK

Etude de la biogenèse et du contrôle qualité des particules ribonucléoprotéiques en utilisant le facteur bactérien Rho comme un outil

Chez les eucaryotes, l'information génétique est transcrite en ARN messager qui subit plusieurs étapes de maturation et évènements d'assemblage avant d'être exporté hors du noyau. Ces modifications du transcrit sont effectuées par de nombreux facteurs protéiques recrutés au transcrit naissant, formant ainsi une particule ribonucléoprotéique (mRNP). La biogenèse du mRNP est étroitement liée avec la transcription et le contrôle qualité afin d'assurer l'efficacité et l'exactitude de la production de mRNPs matures. Des études récentes suggèrent que les membres du complexe THO-Sub2 pourraient être des facteurs cruciaux dans le couplage de la transcription, de la biogénèse du mRNP et de l'export. Dans notre groupe, nous avons mis en œuvre un essai novateur pour étudier la biogénèse du mRNP et le contrôle qualité, basé sur l'expression du facteur Rho bactérien dans *Saccharomyces cerevisiae*. Rho interfère avec l'assemblage adéquat du mRNP et génère des transcrits aberrants qui sont dégradés par la machinerie de dégradation nucléaire. Dans cette étude, nous avons utilisé le système expérimental Rho pour mieux comprendre Rrp6 et l'implication de l'exosome dans la dégradation des transcrits liée au contrôle qualité, ainsi que pour mieux caractériser le rôle et la fonction du complexe THO-Sub2 dans le processus de biogénèse du mRNP. Les résultats obtenus révèlent une différence intéressante dans le comportement des membres du complexe THO sous l'action de Rho et dévoilent leur dépendance à la liaison à l'ARN, ce qui n'aurait pas pu être observé avec d'autres techniques expérimentales. Cela confirme le potentiel attendu du système expérimental basé sur Rho dans l'étude des facteurs protéiques impliqués dans la biogénèse et le contrôle qualité du mRNP.

Mots clés: biogenèse du mRNP, contrôle qualité, THO-Sub2, facteur bactérien Rho, Rrp6

Study of ribonucleoprotein particle biogenesis and quality control by a novel technique using bacterial Rho factor as a tool

In eukaryotes, the genetic information is transcribed into messenger RNA which undergoes various processing and assembly events prior to its export from the nucleus. These transcript modifications are performed by numerous protein factors recruited to the nascent transcript, thus making a messenger ribonucleoprotein particle (mRNP). mRNP biogenesis is tightly interconnected with both transcription and quality control to ensure efficiency and accuracy in production of mature mRNPs. Recent findings suggest that members of THO-Sub2 complex might be crucial factors in coupling transcription, mRNP biogenesis and export. In our group, we have implemented an innovative assay to study mRNP biogenesis and quality control, based on the expression of the bacterial factor Rho in *Saccharomyces cerevisiae*. Rho interferes with proper mRNP assembly and generates aberrant transcripts degraded by the nuclear degradation machinery. In this study, we use Rho experimental system to expand our findings on Rrp6 and exosome involvement in quality control degradation of transcripts, as well as to better characterize the role and function of THO-Sub2 complex in the process of mRNP biogenesis. Obtained results reveal an interesting difference in behavior of THO complex members upon Rho action and disclose their dependence on binding to the RNA, which could not be observed by other experimental techniques. This substantiates the expected potential of Rho-based experimental system in the study of protein factors involved in mRNP biogenesis and quality control.

Keywords: mRNP biogenesis, quality control, THO-Sub2, bacterial factor Rho, Rrp6

Centre de Biophysique Moléculaire
CNRS UPR4301
Rue Charles Sadron
45071 Orléans Cedex 2