Uloga kompleksa Ccr4-Not u transkripciji heterokromatinskih gena u kvasca Schizosaccharomyces pombe

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Graduation Thesis

Zagreb, 2019.

Sveučilište u Zagrebu

Prirodoslovno-matematički fakultet

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Ovaj rad je izrađen u Laboratoriju za ekspresiju i održavanje genoma, u Genskom Centru Sveučilišta Ludwig-Maximilians u Münchenu, pod vodstvom prof. dr. Maria Halića. Rad je predan na ocjenu Biološkom odsjeku Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu radi stjecanja zvanja magistra molekularne biologije.

TEMELJNA DOKUMENTACIJSKA KARTICA

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Proteinski kompleks Ccr4-Not je uključen u regulaciju ekspresije gena u eukariotskim stanicama na više razina, od inicijacije transkripcije do degradacije molekula RNA (Collart, 2016). Duge nekodirajuće molekule RNA (*eng*. long non-coding RNA, lncRNA), koje su transkripti heterokromatinskih sekvenci, također su pod nadzorom ovog kompleksa. U kvasca *Schizosaccharomyces pombe*, nakupljanje lncRNA unutar jezgre rezultira gubitkom heterokromatina i narušava integritet kromosoma; stoga ih je potrebno ukloniti za očuvanje strukture heterokromatina. Prema tome, Ccr4-Not je neophodan za održavanje organizacije heterokromatina, putem razgradnje transkripata lncRNA (Brönner i sur., 2017). Cilj ovog rada je odrediti promjenu u razini prepisivanja heterokromatinskih područja u različitim mutantama podjedinica kompleksa Ccr4-Not te istražiti koji su stanični putevi zahvaćeni kada je ovaj kompleks izvan funkcije. U tu svrhu primijenjena je metoda RNA imunoprecipitacije popraćena sekvenciranjem nove generacije (RIP-seq). Pomoću specifičnog antitijela protiv RNA polimeraze II, transkripti koji potječu od heterokromatina su izolirani i pripremljene su biblioteke RNA. Visoko protočno sekvenciranje biblioteka RNA je izvedeno na platformi Illumina GA IIX. Podaci o sekvenciranju obrađeni su i analizirani različitim bioinformatičkim alatima. Usporedba transkripcije između divljeg tipa i mutantnih sojeva *S. pombe* omogućila je kvantificiranje promjene u prepisivanju heterokromatina, detektiranje gena čija je transkripcija povećana te otkrivanje oštećenih staničnih procesa i metaboličkih puteva u mutantama kompleksa Ccr4-Not.

(36 stranica, 6 slika, 6 tablica, 75 literaturnih navoda, jezik izvornika: engleski)

Rad je pohranjen u središnjoj biološkoj knjižnici.

Ključne riječi: heterokromatin, Ccr4-Not, transkripcija, imunoprecipitacija, sekvenciranje, duge nekodirajuće RNA

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Rad prihvaćen: 18. 09. 2019

BASIC DOCUMENTATION CARD

University of Zagreb
Faculty of Science
Department of Biology

Gradutaion Thesis

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The Ccr4-Not protein complex is involved in the regulation of gene expression in eukaryotic cells at multiple levels, from transcription initiation to degradation of RNA molecules (Collart, 2016). Long non-coding RNA (lncRNA) molecules, which are transcripts of heterochromatic sequences, are also under the control of this complex. In the yeast *Schizosaccharomyces pombe*, lncRNA accumulation within the nucleus results in loss of heterochromatin and impairs chromosome integrity; thus, they must be removed to preserve the structure of heterochromatin. Therefore, the Ccr4-Not complex is essential for the maintenance of heterochromatin organization, via degradation of lncRNA transcripts (Brönner *et al.*, 2017). The aim of this work was to determine the change in the level of transcription of heterochromatic regions in various subunit mutants of the Ccr4-Not complex and to examine which cellular pathways are affected when this complex is out of function. For this purpose, RNA immunoprecipitation followed by next-generation sequencing (RIP-seq) was applied. Using a specific antibody against RNA polymerase II, transcripts originating from heterochromatin were isolated and RNA libraries were prepared. High-throughput sequencing of RNA libraries was performed on the Illumina GA IIX platform. The sequencing data were processed and analyzed by various bioinformatic tools. Comparison of transcription between wild-type and *S. pombe* mutant strains enabled quantification of alteration in heterochromatin transcription, detection of genes whose transcription is increased, and detection of impaired cellular processes and metabolic pathways in mutants of the Ccr4-Not complex.

(36 pages, 6 figures, 6 tables, 75 references, original in: English)

Thesis deposited in the Central Biology Library

Key words: heterochromatin, Ccr4-Not, transcription, immunoprecipitation, sequencing, long non-coding RNA

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Reviewers: Dr. Vlatka Zoldoš, Prof.; Dr. Dijana Škorić, Prof.; Dr. Nada Oršolić, Prof.
Thesis accepted: 18. 09. 2019

Acknowledgments

First, I'd like to thank my parents for their infinite love, encouragement and support, for being there always when I've needed and for keeping me motivated until the very end of my education.

Very special thanks to Prof. Dr. Mario Halić for accepting me to his lab, enabling me to work in an outstanding scientific environment and opening wide the doors of the academic life. It was a great honour to work with and begin my career with such a succesful and renown scientist, of which I will be proud my entire life.

I would also like to thank Dr. Cornelia Brönner for teaching me all the basics of doing reasearch, for her patience during my experiments and for her exceptional guidance throughout my whole work. I really appreciate her help and her struggle for making me try to get the best out of my lab experience.

In addition, I want to thank all the other members of the Halić Lab, especially to Dr. Silvija Bilokapić, Sigrun Jaklin, Dr. Joseph Bartho, Ilaria Ugolini and Luka Salvi for their tips and suggestions for my experiments, and to Ségolène Démolin, Magdalena Olszak and Gouli Ma for their valuable friendship.

I would like to express my gratitude to Prof. Dr. Vlatka Zoldoš for her supervision and for sparing her time and for her very helpful and precious advice, which made this thesis become much easier.

Also, very special thanks to Vlatka Marjan and Sanjica Mihaljević, for their extremely professional work and for caring about my graduation more than I did.

Finally, I'd like to thank all my friends and relatives for their trust and cheering up even in the most difficult times during my study, which was essential for bringing this journey to an end.

This work has been dedicated to my parents and to the victims of the 13th February bus accident in Skopje*

List of Abbreviations

°C	degree celsius	**$MgCl_2$**	magnesium chloride
AcOH	acetic acid	**min**	minute(s)
BCP	1-bromo-3-chloro-propane	**miRNA**	micro RNA
bp	base pairs	**mRNA**	messenger RNA
DEPC	diethyl pyrocarbonate	**msec**	milliseconds
DMSO	dimethyl sulfoxide	**NaOAc**	sodium acetate
DNA	deoxyribonucleic acid	***nat***	noursethecin marker gene
dNTP	deoxyribonucleotide triphosphate	***otr***	centromeric outer repeats
DTT	dithiothreitol	**p42**	pJR1-3×L plasmid backbone (Moreno *et al.,* 2000), digested with *Sph*I and *Bam*HI
EDTA	ethylenediaminetetraacetic acid	**PCR**	polymerase chain reaction
EMM	Edinburgh Minimal Medium	**PNK**	T4 DNA polynucleotide kinase
EMM-leu	Edinburgh Minimal Medium without the amino acid leucine	**poly(A) tail**	3' end of mRNAs
EtOH	ethyl alcohol	**qRT-PCR**	quantitative Real-Time PCR
F	in context of primers: forward	**R**	in context of primers: reverse
fw	forward	**RdRC**	RNA-dependent RNA polymerase complex
h	hour(s)	**rev**	reverse
H1	histone 1	**RITS**	RNA induced transcriptional silencing
H2A	histone 2A	**RNA**	ribonucleic acid
H2B	histone 2B	**RNAi**	RNA interference
H3	histone 3	***S. cerevisiae***	*Saccharomyces cerevisiae*
H4	histone 4	***S. pombe***	*Schizosaccharomyces pombe*
HPH	hygromycin B resistance gene	**SDS**	sodium dodecyl sulfate
kan	kanamycin resistance gene	**sec**	second(s)
kb	kilo base pair	**siRNA**	small interfering RNA
KCl	potassium chloride	**T_M**	melting temperature of dsDNA / primers and DNA
LB medium	Luria-Bertani medium	**TRIS**	2-Amino-2-hydroxymethyl-propane-1,3-diol
LEU2	leucine synthesis gene from *S. cerevisiae*	***ura4***	gene for orotidine 5'-phosphate decarboxylase from *S. pombe*
Leu	leucine	**wt**	wild type
LiOAc	lithium acetate	**YES**	Yeast Extract with Supplements

Contents

1. INTRODUCTION

Regulation of gene expression is fundamental for all biological entities. It is essential to adjust the level of genome activity for cell viability, growth, proliferation and differentiation. Strategies for genome control vary between prokaryotic and eukaryotic organisms. While prokaryotes have relatively simpler organization and management of their genes, eukaryotes have evolved more complex mechanisms for gene tuning.

1.1. Organization of eukaryotic genome

One of the key differences between the prokaryotic and eukaryotic cells is the organization of the DNA within the cell. Genetic material of the prokaryotes is irregularly located within an area known as the nucleoid, where DNA coexists with RNA and nucleoid-associated proteins (Thanbichler *et al.*, 2005). Unlike prokaryotic DNA, which is naked within the cell, DNA of the eukaryotic organisms is wrapped around a particular group of proteins known as histones (Kornberg, 1974). The nucleosome core particle consists of approximately 146-147 base pairs (bp) of DNA wrapped in 1.67 left-handed superhelical turns around a histone octamer, consisting of 2 copies each of the core histones H2A, H2B, H3, and H4 (Luger, 1997). Apart from the octamer exists the linker histone H1. The sequence of H1 is the least conserved of all histones and is not present in *Schizosaccharomyces pombe* (Prieto *et al.*, 2012). This histone is located between nucleosomes and its role includes stabilizing higher levels of chromatin organization (Garcia-Ramirez *et al.*, 1992). Core particles are connected by stretches of linker DNA, which can be up to about 80 bp long. Significant for the higher-order structures of chromatin are the histone tails of the chromatin. Each histone of the octamer has a protruding N-terminal tail which is prone to post-translational modifications (PTMs). The most common PTMs are methylation, acetylation, phosphorylation, ubiquitination and ADP ribosylation (Bowman and Poirier, 2015). Accordingly, the nucleosome not only enables a compact packaging of the DNA inside the nucleus, but also improves genome stability and regulates DNA accessibility (Figure 1.1). As a result, the availability of the DNA sequences to the transcription machinery, and hence the rate of transcription of that sequence changes.

Based on the histone composition and chemical groups attached to them, the eukaryotic genome can be found in two structurally and functionally different states: euchromatin and heterochromatin. Although the initial distinction was made based on the

differential staining of these regions, where euchromatin appears as light-colored bands and heterochromatin stains darkly (Passarge, 1979), today they are distinguished by their properties at the molecular level.

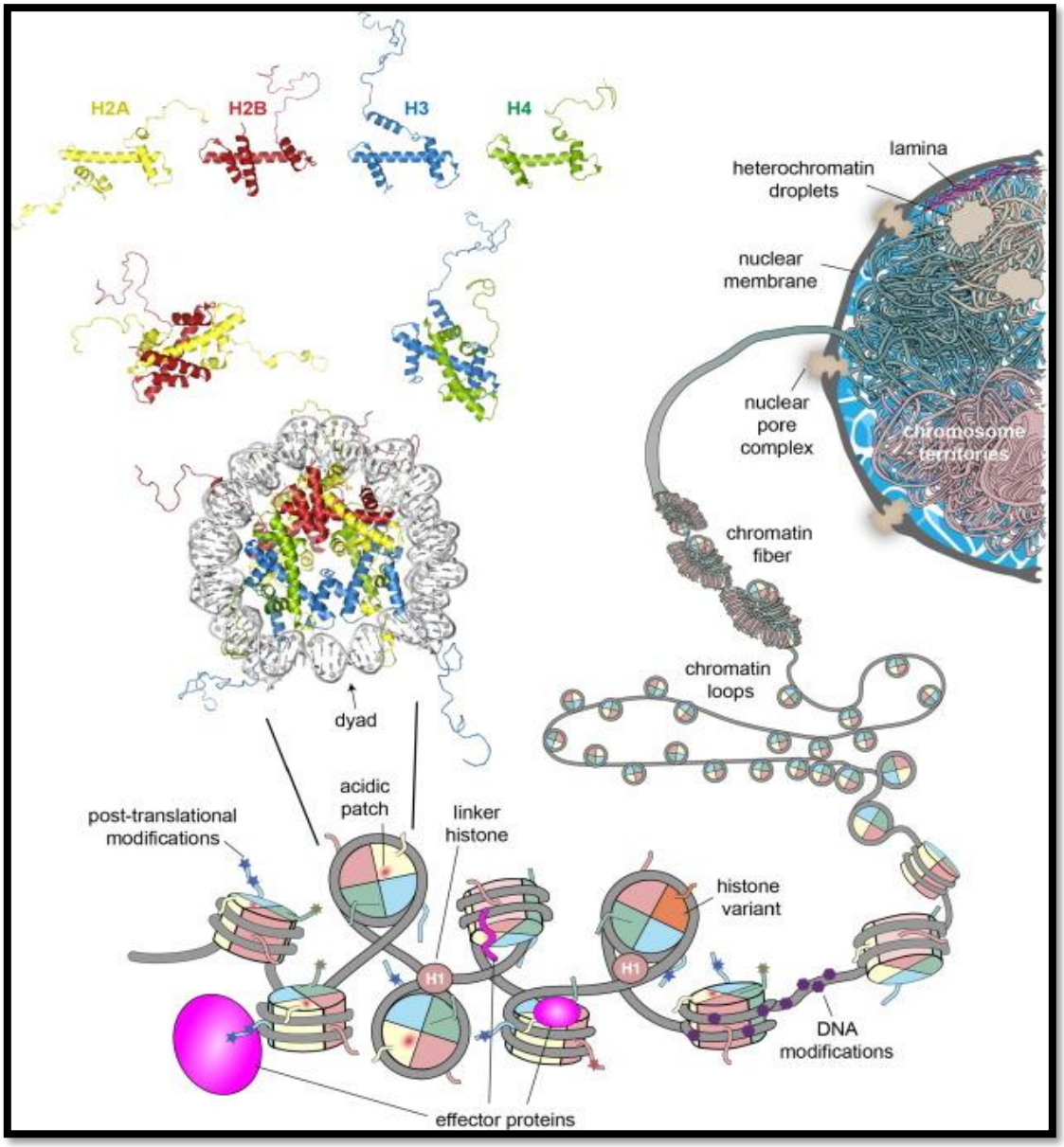

Figure 1.1. DNA organization in eukaryotes. In the top left histone structures can be seen, where histone H3 is shown in blue, H4 in green, H2A in yellow, H2B in red and in DNA in grey. At the bottom, different modifications and interactions of the nucleosome are schematically illustrated. On the right, higher levels of chromatin organization are depicted (van Emmerik and van Ingen, 2019).

1.1.1. Euchromatin

Euchromatin is the loosely packed, gene-rich part of chromatin which is abundantly transcribed (Huisinga *et al.*, 2006). It is characterized by permissive epigenetic marks, such as hyperacetylation of histones at lysine, which are recognized by proteins with a bromodomain,

like chromatin remodelers and transcriptional modifiers (Zeng and Zhou, 2002). Also, methylation of histone 3 at lysine 4 (H3K4) is specific to euchromatin and is associated with increased transcription (Tamaru, 2010).

1.1.2. Heterochromatin

Tightly packed chromatin regions, which stain intensely and are localized to the periphery of the nucleus represent the heterochromatin. Compared to euchromatin, it is more condensed, gene-poor and transcriptionally passive (Huisinga *et al*., 2006). Typical features of heterochromatin are hypoacetylated histone tails, histone 3 lysine 9 di- and trimethylation, association of the heterochromatin protein 1 (HP1) and methylation of the DNA at cytosine residues (5mC) in higher eukaryotes showing this modification (Tamaru, 2010). In contrast to higher eukaryotes, fission yeast lacks DNA methylation (Wilkinson *et al.*, 1995). Thus, silencing in *S. pombe* mainly is achieved through chromatin modifications and RNA interference machinery.

On the other hand, heterochromatin is also found at developmentally regulated loci, where the chromatin state can change in response to cellular signals and gene activity. These regions are referred to as facultative heterochromatin (Grewal and Jia, 2007).

1.2. *Schizosaccharomyces pombe* as a model organism

S. pombe is a unicellular, rod-shaped ascomycete of the kingdom Fungi. Due to its typical mode of reproduction by medial division, it is also known as the fission yeast. In 2002, it became the sixth eukaryotic model organism whose genome was sequenced (Wood *et al.*, 2002). Its genome consists of 13.8 Mb distributed between 3 relatively large chromosomes (Figure 1.2) of 5.6, 4.8 and 3.5 Mb respectively for Chromosomes I, II and III compared to 16 smaller chromosomes in *Saccharomyces cerevisiae*. According to the model organism database (MOD) PomBase, there are currently 5064 protein-coding genes reported (https://www.pombase.org/status/statistics). Of the 5064 known or predicted fission yeast proteins, at this time, 2154 have a published biological role, 2050 have a biological role inferred from an experimentally characterized ortholog (usually from budding yeast), and 850 have no known biological role [inferred from the absence of Gene Ontology (GO) biological process annotation] (Hoffman *et al*., 2015).

What makes it an ideal model organism is its minimalistic nature as a eukaryote – it is unicellular and has a fast replication time, which enables easy handling and conducting large number of experiments. In addition, it has a small genome which is similar to higher eukaryotes that makes it a perfect candidate for genetic studies.

1.3. Heterochromatin in *S. pombe*

The fission yeast *Schizosaccharomyces pombe* has well characterized regions of heterochromatin and has proven to be a powerful model for elucidation of epigenetic silencing mechanisms (Goto and Nakayama, 2012). Both types of heterochromatin, facultative and constitutive, exist in fission yeast. Facultative heterochromatin can be found at so called heterochromatic islands which are present in vegetative cells and contain clusters of genes that are expressed in meiotic cells (Zofall *et al.*, 2012). On the other hand, constitutive heterochromatin of the *S. pombe* genome forms at the pericentromeric repeats, telomeres and the mating-type region (Figure 1.2) (Allshire and Ekwall, 2015). Each of these regions is significant for the cell in a different way.

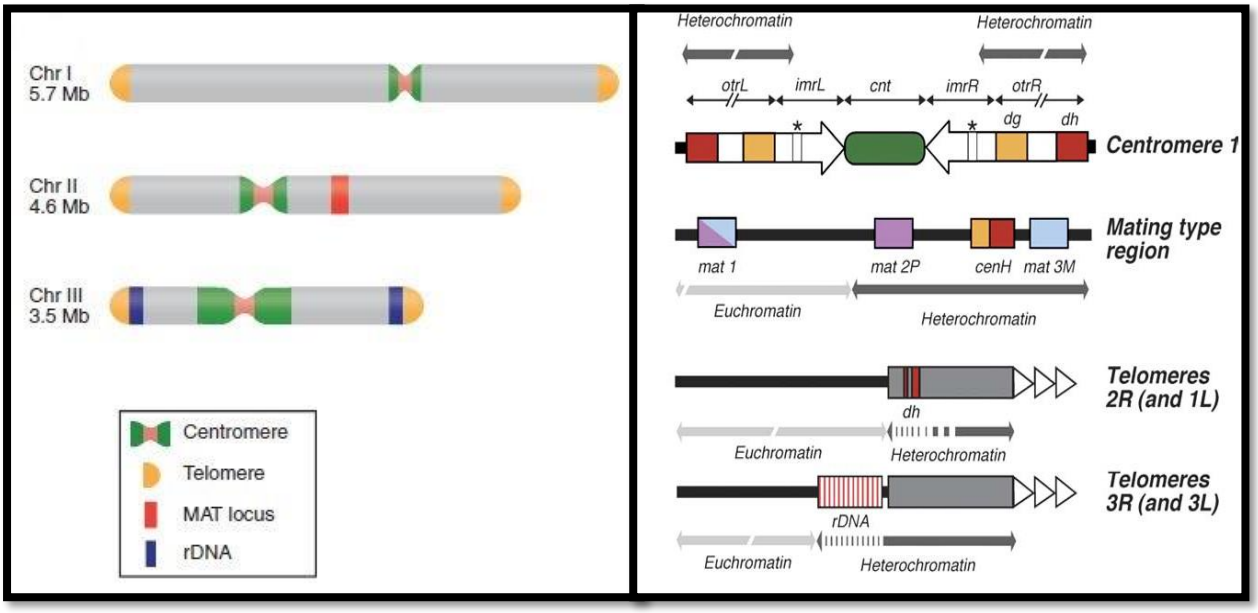

Figure 1.2. Schematic representation of *S. pombe* chromosomes. On the left, the three chromosomes are depicted showing the four main regions of heterochromatin: centromere, telomere, mat2/3, and rDNA regions (Allshire and Ekwall, 2015). On the right, a detailed organization of the heterochromatic regions is shown (Verdel and Moazed, 2005).

1.3.1. Centromeric heterochromatin

Each chromosome of the fission yeast contains a centromere (chromosomal locus where kinetochores assemble), which are flanked by inverted repeats like in higher eukaryotes. The central core (*cen*) is surrounded by the inner most repeats (*imr*), which are unique for each chromosome. They are followed by the outer repeat regions (*otr*) which consist of two repeating units, *dg* and *dh*, whose copy numbers vary between the chromosomes (Martienssen *et al.*, 2005; Wood *et al.*, 2002). The *imr* and *otr* sequences are the places where centromeric heterochromatin is established. RNA interference (RNAi) pathway is essential for centromeric heterochromatin formation and maintenance (Volpe *et al.*, 2002).

1.3.2. Heterochromatin in the mating-type region

Heterochromatin in the mating type region is crucial for *S. pombe*, since it determines its sexual life cycle (Allshire and Ekwall, 2015). Mating-type region is found on the right arm of chromosome 2 (Figure 1.2), consisting of three components: *mat1*, *mat2-P* and *mat3-M*. Fission yeast cells switch mating type by homologous recombination where the information in the expressed *mat1* locus is replaced with information copied from one of two silent loci, *mat2* or *mat3* (Klar, 2007).

Silent information for the P and M mating types is stored at respectively *mat2* ~17 kb centromere-distal to *mat1*, and *mat3* ~29 kb centromere-distal to *mat1*. The mating-type specific information at *mat1*, *mat2* and *mat3* is flanked by short homology boxes, the centromere-distal H1 box and the centromere-proximal H2 box (Kelly *et al.*, 1988). Other elements are specific for *mat2* and *mat3*. *mat2* and *mat3* are furthermore embedded in a ~20 kb heterochromatic domain that spans the *mat2-mat3* interval and extends on both sides to inverted repeat boundaries (Jakočiūnas *et al.*, 2013).

This domain has been studied extensively. It provides one of the best characterized model systems for how heterochromatic regions can be established and maintained. In this domain, histones are hypoacetylated, histone H3 is methylated at lysine 9 (H3K9me) in an RNAi-dependent manner, and chromodomain proteins of the HP1 family are associated with the modified histones (Noma *et al.*, 2001).

1.3.3. Subtelomeres

Subtelomeres of the fission yeast lie on both of arms of the chromosomes 1 and 2, with several copies of *tlh* in between. There is a slight difference, however, on chromosome 3 – it contains ribosomal DNA (rDNA) tandem repeats embedded between euchromatic genes and telomeres with H3K9 methylation within the rDNA open reading frames (Figure 1.2) (Cam *et al.*, 2005). Heterochromatin is established such that the SHELTERIN complex binds telomeres and in turn recruits the methyltransferase complex CLRC and the deacetylase complex SHREC (Kanoh *et al.*, 2005; Sugiyama *et al.*, 2007; Motamedi *et al.*, 2008). Consequently, CLRC and SHREC complexes move from the telomeric repeats towards the subtelomeric region to establish heterochromatin. Besides the SREC complex, RNAi too, is involved in the establishment of subtelomeric heterochromatin, which is lost only if both SHREC and RNAi pathways are eliminated (Kanoh *et al.*, 2005; Hansen *et al.*, 2006). Furthermore, depletion of the telomeric repeats, where the Shelterin complex binds, until the subtelomeres and together with the *tlh*, causes loss of heterochromatin (Kanoh *et al.*, 2005), showing that both pathways are recruited to these regions. Sequence of the tlh gene shares a homologous region with cenH, which is a source of siRNA in wild type cells (Cam et al., 2005), suggesting that with the loss of *tlh*, RNAi recruitment is impaired. Overexpression of *tlh* increases the viability of telomerase deficient cells, but the function of the annotated RecQ type helicase remains unknown (Mandell et al., 2005). Even though several stress induced genes are located at the subtelomeric region, *tlh* expression is not influenced by nitrogen starvation or growth in stationary phase (Hansen *et al.*, 2005). Overall, subtelomeric heterochromatin is important for maintaining genome stability via prevention of inter- and intrachromosomal recombination or end fusion (Kanoh *et al.*, 2005).

1.4. RNAi and RNA Pol II machinery in heterochromatin formation

RNA interference (RNAi) is one of the key mechanisms involved in heterochromatin formation in *S. pombe*. Comparative genomics studies have shown that RNAi has evolved from an ancestral role in transposon silencing to its role in *S. pombe* heterochromatin assembly (Rhind *et al.*, 2011).

Initiation of transcription, transcriptional elongation and transcript processing are as important for heterochromatin assembly as they are for euchromatic gene expression (Allshire and Ekwall, 2015). Hence, it is important to explore the involvement of RNA Pol II in heterochromatin assembly. As one of the central players of heterochromatin formation, RNA Pol II was a promising target for transcription analysis conducted in this work.

1.5. The Ccr4-Not complex

Carbon **c**atabolite **r**epression **4** – **n**egative **o**n **T**ATA-less (Ccr4-Not) is a versatile protein complex present in all eukaryotes, which is involved in gene regulation at multiple levels, from transcription initiation to RNA degradation (Collart, 2016). It consists of up to 10 subunits with various functions (Cotobal *et al.*, 2015). The most detailed information to date on the molecular architecture of the entire complex is a medium-resolution (20 Å) structure obtained through cryo-EM (Figure 1.3) (Ukleja *et al.*, 2016). Namely, Not1 (**n**egative **o**n **T**ATA **1**) is the largest (237 kDa) and represents the scaffold of this macromolecular assembly. Ccr4 (**C**arbon **c**atabolite **r**epression **4**; 76 kDa) and Caf1 (**C**cr4-**a**ssociated **f**actor **1**; 37.5 kDa) are the deadenylase subunits of the complex. Not4 RING E3 ligase (Mot2 in *S. pombe*; 54 kDa) carries out the second enzymatic activity, ubiquitination (Collart, 2013). The remaining subunits, Not2, Not3 and Not5 (34, 73 and 66 kDa, respectively), form the so called Not module, with no clear function yet assigned (Bhaskar *et al.*, 2013). In addition, Caf40 (**C**cr4-**a**ssociated **f**actor **40**; Rcd1 in *S. pombe*; 32 kDa) reportedly has a role in RNA degradation, as part of the miRNA machinery (Chen *et al.*, 2014). Still, the composition of the complex varies between species. For instance, Caf130, which is an additional subunit of the complex in *Saccharomyces cerevisiae*, is absent from the fission yeast. In *S. pombe*, moreover, Mmi1 stably interacts with the complex to preserve heterochromatin integrity at meiotic genes (Cotobal *et al.*, 2015).

The main function of the Ccr4-Not complex is RNA degradation. In higher eukaryotes it is suggested that the BTG/TOB family of proteins direct the complex to mRNAs for generic digest (Winkler, 2010). These proteins are not conserved in yeast, proposing another general recruitment mechanism (Collart, 2016). Furthermore, the Ccr4-Not complex is connected to specific RNA decay. In higher eukaryotes, RNAi targets particular mRNA using sRNA called micro RNA (miRNA) (Huntzinger and Izaurralde, 2011). Via the interaction of CNOT9 (Rcd1 in *S. pombe*) with the GW182 protein (Tas3 in *S. pombe*) of the RITS complex, the Ccr4-Not complex is recruited to degrade those transcripts (Chen *et al.*, 2014; Mathys *et al.*, 2014). Additionally, Tristetraprolin (TTP) was published to mediate Ccr4-Not dependent decay of specific, AU rich element (ARE) containing mRNAs (Fabian *et al.*, 2013; Sandler *et al.*, 2011) and also the germline specific protein Nanos was shown to interact with the Ccr4-Not complex (Suzuki *et al.*, 2010). Besides deadenylation, the Ccr4-Not complex relates to protein turnover by the E3 ubiquitin ligase Not4 (=Mot2 in *S. pombe*) (Dimitrova *et al.*, 2009; Laribee *et al.*, 2007; Matsuda *et al.*, 2014; Mersman *et al.*, 2009). Additionally, the Ccr4-Not complex is associated with regulation of transcription (Collart, 2016; James *et al.*, 2007; Kruk *et al.*, 2011), but the precise role remains elusive.

Current data suggest that heterochromatic silencing is a combination of transcriptional silencing and RNA degradation. The heterochromatin structure is considered to reduce RNA Pol II occupancy (Chen and Widom, 2005; Schuettengruber *et al.*, 2007) and to recruit the RNA degradation machinery to degrade nascent transcripts (Brönner *et al.*, 2017; Bühler *et al.*, 2008; Cotobal *et al.*, 2015; Marasovic *et al.*, 2013; Pisacane and Halic, 2017; Reyes-Turcu *et al.*, 2011; Reyes-Turcu and Grewal, 2012; Sugiyama *et al.*, 2016). How transcriptional silencing and RNA degradation pathways corroborate and how much each pathway contributes to silencing is not known.

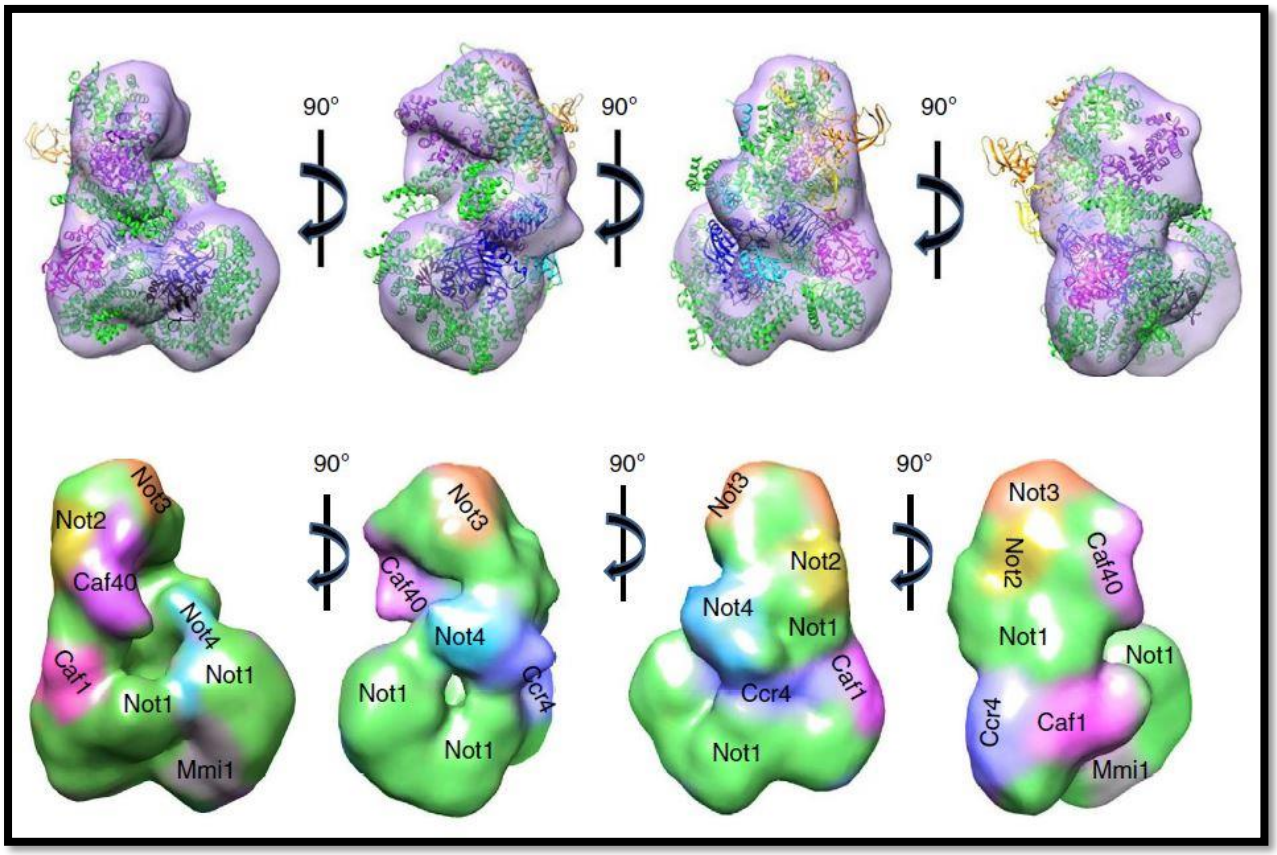

Figure 1.3. Architecture of the Ccr4-Not complex in *S. pombe* (Ukleja *et al.*, 2016). Reconstruction of the Ccr4-Not complex and indication of possible subunit localization of the Ccr4-Not complex. The Ccr4-Not complex is L-shaped and consists of the core proteins Not1, Not2, Not3, Rcd1 (=Caf40), Caf1, and Ccr4. In *S. pombe*, Not4 (=Mot2) and Mmi1 are also stably associated with the complex.

1.6. Aim of the graduation thesis

The main objective of this graduation thesis was to determine the change in the level of transcription of heterochromatic regions in various subunit mutants of the Ccr4-Not complex and to examine which cellular pathways are affected when this complex is out of function. The contribution of these pathways to heterochromatic silencing in fission yeast cells was quantified by analyzing RNA Polymerase II occupancy, nascent RNA and steady state RNA levels. In addition, using these data, how subunits of the Ccr4-Not complex contribute to transcriptional silencing and/or RNA degradation was defined.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. *Schizosaccharomyces pombe* strains

All *S. pombe* strains used throughout this thesis are listed in Table 2.1. Construction of the mutant strains is described in experimental procedures.

Table 2.1. *S. pombe* strains used in this work. Greek delta letter (Δ) indicates deletion of the corresponding gene and asterisk (*) symbolizes the activity mutants of the selected strains.

Strain	Numeric label	Genotype of the strain
wild type	63	h+ otrR(SphI)::ura4 ura4 DS/E leu1-32 ade6-M210
	65	h+ otr1R(SphI)::ura4+ ura4-DS/E leu1-32 ade6-M210 natMX6::3xFLAG-ago1
clr4Δ	80	h+ leu1-32 ade6-210 ura4DS/E otrR(SPhI)::ura4+ clr4Δ::kanMX6
caf1Δ	510	h+ otr1R(SphI)::ura4+ ura4-DS/E leu1-32 ade6-M210 nat::FLAG-Ago1 ura4::otr Δcaf1::kan
	594	h+ otr1R(SphI)::ura4+ ura4 DS/E leu1 32 ade6 M210 nat::FLAG Ago1 ura4::otr Δcaf1::kan
ccr4Δ	544	h90 otr1R(SphI)::ura4+ ura4-DS/E leu1-32 ade6-M210 natMX6::3xFLAG-ago1 ccr4Δ::hphMX6
caf1*	1113	TEL72 h90 (tel(1L)::his3 tel(2L)::ura4 otr1R::ade6) gen reintegration nat::caf1promoter-caf1D53AD243AD174A
ccr4*	1167	TEL72 h90 (tel(1L)::his3 tel(2L)::ura4 otr1R::ade6) 588 x 1140 ccr4H664A – ccr4T::kan
caf1*ccr4*	1168	TEL72 h90 (tel(1L)::his3 tel(2L)::ura4 otr1R::ade6) 588 x 1140 nat::caf1promoter-caf1D53AD243AD174A; ccr4H664A – ccr4T::kan

2.1.2. Chemicals and consumables

Chemicals and consumables used in this work are listed in Table 2.

Table 1.2. List of chemicals and their manufacturers.

Chemical substance	Supplier
1-Chloro-3-Bromo-Propane	Sigma Aldrich (Steinheim, Germany)
Adenine	Formedium™ (Hunstanton, United Kingdom)
Agarose Ultra Pure	Invitrogen™ (Carlsbad, USA)
Ampicillin	Formedium™ (Hunstanton, United Kingdom)
Bacto™ Agar	Beckton, Dickinson & Company (Heidelberg, Germany)
DEPC	Roth (Karlsruhe, Germany)

DMSO	New England Biolabs (Frankfurt, Germany)
dNTPs	Metabion (Martinsried, Germany)
DTT	Invitrogen™ (Carlsbad, USA)
EDTA VWR	(Darmstadt, Germany)
Ethanol	Roth (Karlsruhe, Germany)
GeneRuler™ 1 kb DNA Ladder Mix	Thermo Scientific (Waltham, USA)
Glucose	Formedium™ (Hunstanton, United Kingdom)
Glycerol	Roth (Karlsruhe, Germany)
Glycogen	Thermo Scientific (Waltham, USA)
Histidine	Formedium™ (Hunstanton, United Kingdom)
Isopropanol	VWR (Darmstadt, Germany)
KCl	Merck (Darmstadt, Germany)
Leucine	Formedium™ (Hunstanton, United Kingdom)
Lysine	Formedium™ (Hunstanton, United Kingdom)
MgCl2	Merck (Darmstadt, Germany)
N2, liquid	Linde (München, Germany)
NaCl	Merck (Darmstadt, Germany)
NaOAc	Merck (Darmstadt, Germany)
NaOH	Roth (Karlsruhe, Germany)
PEG 3350	Sigma Aldrich (Steinheim, Germany)
Phenol:Chloroform:Isoamyl alcohol	Invitrogen™ (Carlsbad, USA)
RNasin®	Promega (Mannheim, Germany)
Salmon Sperm DNA Sodium Salt	Roth (Karlsruhe, Germany)
SDS	SERVA (Heidelberg, Germany)
Sorbitol	Roth (Karlsruhe, Germany)
SP Supplements	Formedium™ (Hunstanton, United Kingdom)
SybrGreen I	Biozym Scientific GmbH (Oldendorf, Germany)
SybrGreen II	Biozym Scientific GmbH (Oldendorf, Germany)
TRI Reagent® Solution	Ambion® (Austin, USA)
Tris Pufferan®	Roth (Karlsruhe, Germany)
Triton X-100	Roth (Karlsruhe, Germany)
Uracil	Formedium™ (Hunstanton, United Kingdom)
Yeast Extract	Formedium™ (Hunstanton, United Kingdom)

2.1.3. Buffers and solutions

All the solutions used in this work were prepared from analytical grade reagents supplied by the manufacturers, as listed in Table 2. Solutions and buffers (Table 2.3) were made using deionized water and sterilized by autoclaving or filtration. Solutions used in RNA experiments were prepared and stored in sterilized glassware, treated overnight with 0.1% (v/v) diethyl pyro carbonate (DEPC) and then autoclaved. Commercial enzymes and their corresponding buffers were obtained from the suppliers listed in Table 2.4.

Table 2.2. Solutions and buffers

YES (Yeast Extract with Supplements)	
• **Liquid Medium**	
Yeast Extract	5 g/l
Glucose	30 g/l
Leucine	225 mg/l
Adenine	225 mg/l
Uracil	225 mg/l
Histidine	225 mg/l
Lysine	225 mg/l
• **Solid Medium (contains additionally)**	
Bacto® Agar	20 g

1× Taq Buffer	
Tris-HCl	10 mM
KCl	50 mM
$MgCl_2$	1.5 mM
pH	8.3

5× Loading Dye for DNA	
Glycerol 50 %	900 µl
Bromphenol Blue	100 µl
SybrGreen® I	2 µl

1× Lysis Buffer for DNA isolation for *S. pombe*	
NaOAc pH 5.2	300 mM
EDTA	10 mM
SDS (v/v)	1 %

1× TAE buffer	
Tris	40 mM
AcOH	20 mM
EDTA (pH 8.0)	1 mM

Table 2.3. Commercial enzymes and buffers

Enzyme	Supplier
DNase I recombinant, RNase free	Roche (Basel, Switzerland)
***Phusion* DNA Polymerase**	Halić lab
RNase A	Invitrogen™ (Karlsruhe, Germany)
Buffer	**Supplier**
DNase I recombinant, RNase free	Incubation Buffer Roche (Basel, Switzerland)
First Strand Buffer 5X	Invitrogen™ (Karlsruhe, Germany)

2.1.4. Equipment and instruments

Laboratory equipment, commercial kits and other instruments utilized for conducting the experiments have been listed in Table 2.5.

Table 2.4. List of equipment and other tools used for the experiments.

Equipment	Manufacturer
Centrifuge 5424	Eppendorf (Hamburg, Germany)
FlexCycler²	Analytik Jena (Jena, Germany)
MicroPulser Electroporator	Bio-Rad (München, Germany)
Gel electrophoresis	Bio-Rad (München, Germany)
NanoDrop® ND-1000	PEQ Lab (Erlangen, Germany)
Primus 96 advanced	PEQ Lab (Erlangen, Germany)
TOptical Thermocycler	Biometra (Göttingen, Germany)
Rotanta 460R	Hettich Lab Technology (Tuttlingen, Germany)
Thermomixer comfort	Eppendorf (Hamburg, Germany)
Kits	**Manufacturer**
NucleoSpin Plasmid Quick Pure Kit	Macherey-Nagel (Düren, Germany)
Agencourt AMPure XP Beads	Beckman Coulter (Krefeld, Germany)
QIAquick PCR Purification Kit	QIAGEN (Hilden, Germany)
NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina®	New England Biolabs (Frankfurt, Germany)
NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®	New England Biolabs (Frankfurt, Germany)
Other	**Manufacturer**
96 well for qPCR	4titude® Ltd (Berlin, Germany)
Multiply® -µStrip 0.2 ml chain	Sarstedt AG & Co. (Nümbrecht, Germany)
SafeSeal-Tips Professional (10, 20, 200, 1250 µl)	Biozym Scientific GmbH (Oldendorf, Germany)
SafeSeal tube 1.5 ml	Sarstedt AG & Co. (Nümbrecht, Germany)
Standard Tips	Sarstedt AG & Co. (Nümbrecht, Germany)
Serological Pipette 5 ml/ 10ml/ 25 ml	Sarstedt AG & Co. (Nümbrecht, Germany)

2.2. Methods – Experimental procedures

2.2.1. Strain construction

All the *S. pombe* strains used throughout this work are listed in Table 2.1. Point mutations for Caf1D53AD243AD174A were chosen according to Jonstrup *et al*. (2007). The Ccr4 activity mutant, Ccr4H665A, was taken corresponding to the homologous Ccr4H818A mutant in *S. cerevisiae* (Chen *et al.*, 2002).

Overnight cultures of 50 ml were grown in liquid YES medium to an optical density (OD600) of 0.5. Cells were harvested by centrifugation with 3500 rpm at 20 °C for 5 minutes

and then washed with ice-cold autoclaved water. The cells were quickly spun down and the supernatant was discarded. The pellet was resuspended in 1.2 M sterile ice-cold sorbitol and transferred to 1.5 mL Eppendorf tube. Cells were pelleted with 3500 rpm for 2 minutes at room temperature and then resuspended in 100 µl 1.2 M sterile ice-cold sorbitol. Plasmid DNA of 500 ng was added for transformation. The whole content was transferred to an electroporation cuvette and chilled on ice. Electroporation was performed with the MicroPulser Electroporator by using the ShS program for *S. pombe* with 2.0 kV for 5 milliseconds. Ice-cold sorbitol of 1.2 M was added to the cells immediately. Electroporated cells were plated on selective EMM-Leu plates and incubated at 32 °C for 2-7 days.

Successfully transformed cells were prepared for long term storage from fresh overnight cultures by adding glycerol to a final concentration of 25 %. Cells were flash-frozen immediately in liquid nitrogen and stored at -80 °C.

2.2.2. Total RNA isolation

Total RNA was isolated from a 2 ml (qRT-PCR) yeast culture with OD600 of 1.0 applying the hot phenol method (Wecker, 1959). The pellet was resuspended in 500 µl lysis buffer (300 mM NaOAc pH 5.2, 10 mM EDTA, 1% SDS) and 500 µl phenol-chloroform-isoamylalcohol (25:24:1, Roth) and incubated at 65 °C for 10 min with constant mixing. The organic and aqueous fractions were separated by centrifugation at 20000 x g for 10 minutes. Nucleic acids in the aqueous fraction were precipitated with ethanol and then treated with DNase I (Thermo Scientific) for 1 h or 2 h at 37 °C. DNase was removed by a second phenol-chloroform-isoamylalcohol extraction and ethanol precipitation.

2.2.3. Total RNA and poly(A) RNA sequencing

rRNA of 1 µg total RNA was degraded with Terminator nuclease (Epicentre) in buffer A at 30°C for 2 h. For p(A) RNA sequencing, poly-adenylated RNA was extracted from total RNA with oligo d(T)25 magnetic beads (NEB). The RNA library was obtained using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB). Single end sequencing was performed on an Illumina GAIIX sequencer at the LAFUGA core facility of the Gene Center, Munich. The Galaxy platform was used to demultiplex the obtained reads with Je-Demultiplex-Illu (Goecks *et al.*, 2010).

2.2.4. Chromatin immunoprecipitation (ChIP)

Yeast cultures of 50 ml with an OD600 of 1.2 were cross-linked with 1% formaldehyde (Roth) for 15 min at room temperature. The reaction was quenched with 125 mM glycine for 5 min. The frozen pellet was resuspended in 500 µl lysis buffer (250 mM KCl, 1x Triton-X, 0.1% SDS, 0.1% Na-Desoxycholate, 50 mM HEPES pH 7.5, 2 mM EDTA, 2 mM EGTA, 5 mM MgCl2, 0.1% Nonidet P-40, 20% Glycerol) with 1 mM PMSF and Complete EDTA free Protease Inhibitor Cocktail (Roche). Lysis was performed with 0.25-0.5 mm glass beads (Roth) and the BioSpec FastPrep-24 bead beater (MP-Biomedicals), 8 cycles at 6.5 m/s for 30 s and 3 min on ice. DNA was sheared by sonication (Bioruptor, Diagenode) 35 times for 30 s with a 30 s break. Cell debris was removed by centrifugation at 13000 x g for 15 min. The crude lysate was normalized based on the RNA and Protein concentration (Nanodrop, Thermo Scientific) and incubated with 1.2 µg immobilized (Dynabeads Protein A or G, Thermo Scientific) antibody against dimethylated H3K9 (H3K9me2, abcam AB1220), for at least 2 h at 4 °C. The resin with immunoprecipitates was washed five times with each 1 ml of lysis buffer and eluted with 150 µl of elution buffer (50 mM Tris HCl pH 8.0, 10 mM EDTA, 1% SDS) at 65 °C for 15 min. Cross-linking was reversed at 95 °C for 15 min and subsequent RNase A (Thermo Scientific) digest for 30 min followed by Proteinase K (Roche) digest for at least 2 h at 37 °C or overnight at 65 °C. DNA was recovered by phenol-chloroform-isoamylalcohol (25:24:1, Roth) extraction with subsequent ethanol precipitation. DNA levels were quantified by qRT-PCR and normalized to *tdh1* background levels. Oligonucleotides used for quantification are listed in Table 2.6. For sequencing, a ChIP-seq library was made using the NEBNext Ultra II DNA Library Prep Kit for Illumina kit (NEB). Single end sequencing was performed on an Illumina GAIIX sequencer at the LAFUGA core facility of the Gene Center, Munich. The Galaxy platform was used to demultiplex the obtained reads with Je-Demultiplex-Illu (Goecks *et al.*, 2010).

Table 2.5. Oligonucleotides used in this thesis. Labels indicate internal numbering of the laboratory. (F) is for forward primer and (R) for reverse primer. All primers were synthetized by Metabion (Martinsried, Germany).

Binding site	Label	Sequence (5' → 3')
subtelomeric *tlh1*	179 (F)	CCAGCTCTTTCGTTCAGGAC
	179 (R)	AGTTGACGCTCCTTGGAAGA
centromeric *dg* repeat	110f (F)	CTGCGGTTCACCCTTAACAT
	110f (R)	CAACTGCGGATGGAAAAAGT
tdh1	110a (F)	CCAAGCCTACCAACTACGA
	110a (R)	AGAGACGAGCTTGACGAA
fbp1	110d (F)	TACTGCGATGAAGTCGAACG
	110d (R)	TTGACACGATGACCTGTGGT

act1	219 (F)	GATTCTCATGGAGCGTGGTT
	219 (R)	CTCATGAATACCGGCGTTTT
***SPRRNA.47* (28S rRNA)**	113C (F)	TTTTCTCCTTCTCGGGGATT
	113D (R)	AACACCACTTTCTGGCCATC

2.2.5. RNA immunoprecipitation (RIP)

RNA IP was performed like ChIP but without RNase A digest, with anti-RNA polymerase II CTD repeat YSPTSPS antibody [8WG16] (ab817, abcam). Cells were also crosslinked with 1% Formaldehyde for 15 min. After phenol-chloroform-isoamylalcohol extraction, DNA was digested with DNase I (Thermo Scientific) for 2 h at 37 °C. RNA was recovered with a second phenol-chloroform-isoamylalcohol purification and ethanol precipitation. RNA was either taken for making a sequencing library using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB) or it was reverse transcribed into cDNA with specific primers with subsequent qRT-PCR.

2.2.6. Chromatin fractionation

The frozen pellet of a 10 ml culture with an OD600 of 1.0 was resuspended in 250 µl lysis buffer (250 mM KCl, 1x Triton-X, 0.1% SDS, 0.1% Na-Desoxycholate, 50 mM HEPES pH 7.5, 2 mM EDTA, 2 mM EGTA, 5 mM MgCl2, 0.1% Nonidet P-40, 20% Glycerol) and lysed with 0.25-0.5 mm glass beads (Roth) and the BioSpec FastPrep-24 bead beater (MP-Biomedicals), 8 cycles at 6.5 m/s for 30s and 3 min on ice. (Under the microscope it was analyzed that 99% of the cells were broken.) The lysate was spun at 21000 x g for 20 min and 200 µl of the supernatant were taken as “unbound” fraction. Residual supernatant was removed by washing twice with 800 µl lysis buffer and centrifugation at 21000 x g for 10 min. The pellet was resuspended in 250 µl lysis buffer. The “chromatin” fraction was built of 200 µl of the suspension. The fractions were divided in half, respectively, to separate between RNA and DNA. The DNA samples were treated with RNaseA and Proteinase K before Phenol-Chloroform-Isoamylalcohol treatment and Ethanol precipitation. qRT-PCR was performed without normalization of DNA amount to analyze if chromatin fractionation worked. RNA was recovered by Phenol-Chloroform-Isoamylalcohol treatment, Ethanol precipitation and DNase digest like described for total RNA isolation. Reverse transcription was performed with 100 ng RNA for each sample and specific primers for *tlh* and *fbp1*. qRT-PCR was performed using also non-reverse transcribed sample as control to be sure that no DNA was amplified. In each

fraction, *tlh* RNA was normalized to *fbp1* RNA and presented as fold change compared to "wild type unbound".

2.2.7. Analysis of sequencing data

Single end sequencing of libraries was performed on an Illumina GAIIX sequencer at the LAFUGA core facility of the Gene Center, Munich. The Galaxy platform was used to demultiplex the obtained reads with Je-Demultiplex-Illu (Goecks *et al.*, 2010). Demultiplexed illumina reads were mapped to the *S. pombe* genome, allowing 2 nucleotides mismatch to the genome using Novoalign (htttp://www.novocraft.com). h90 *S. pombe* genome was assembled using the *mat* sequence from Pombase and imported it in IGV. Small RNA Reads mapping to multiple locations were randomly assigned. Using various Perl scripts, the datasets were normalized to the number of reads per million (rpm) sequences for small RNA-seq or reads per million mapping to coding sequences for total RNA-seq, p(A) RNA-seq, ChIP-seq. ChIP data were either normalized by rpm if variation in read amounts was low, if centromeric heterochromatin was lost for example, ChIPseq data were normalized to regions which were not changed in different mutants. Caf1 ChIP reads were summed in a window of 100 nt and divided by a corresponding control to display the fold-change using the Integrative Genomics Viewer (IGV) (http://www.broad.mit.edu/igv). Sequencing data were done in two replicates or the data were confirmed by another method like qRT-PCR. Sequenced strains are listed in Table 2.1.

3. RESULTS

3.1. Transcriptional silencing and degradation of heterochromatic RNA in wild type cells

Primarily, in order to distinguish the contribution of transcriptional silencing and RNA degradation to heterochromatic silencing, S2P-RNA Polymerase II occupancy (S2P-RNA Pol II-bound DNA), nascent elongating RNA (S2P-RNA Pol II-bound RNA), and steady state RNA (polyadenylated RNA (pA) RNA) at heterochromatic and euchromatic regions in *S. pombe* wild type cells were analyzed. For this purpose, how heterochromatin affects the accessibility of RNA Polymerase II was examined, by comparing RNA Pol II occupancy in wild type cells with the occupancy in cells having a deletion of the H3K9 methyltransferase Clr4 (Figure 3.1A, 3.1B).

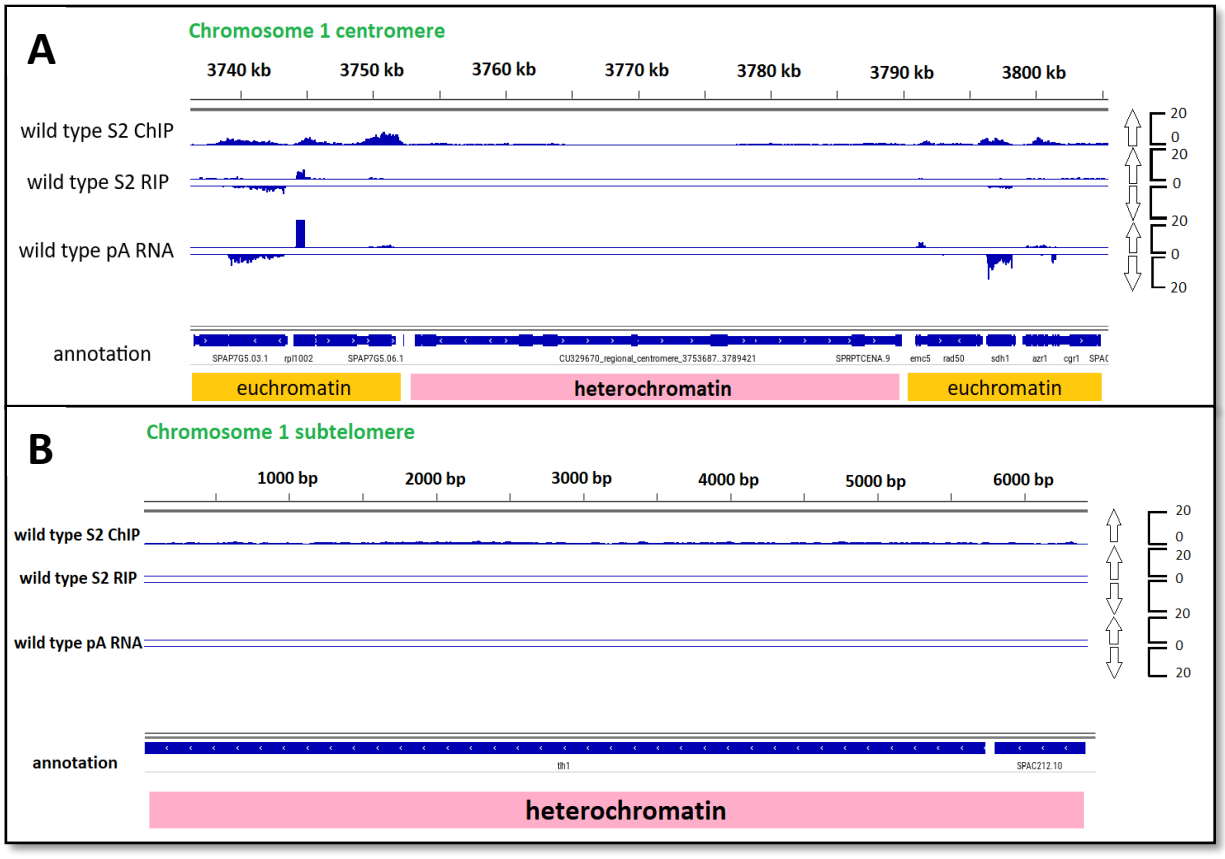

Figure 3.1. Analysis of the next-generation sequencing data showing occupancy of S2-phosphorylated RNA Pol II (ChIP-seq), nascent RNA (S2P- Pol II RIP-seq) and steady state RNA levels (pA RNA-seq) at centromeric (A) and subtelomeric (B) regions in *S. pombe* wild type cells. Locations of genes are indicated as boxes below the coverage according the color code: yellow = protein coding; pink = *dg, dh* (A), *tlh, SPAC212.10* (B).

*clr4*Δ cells do not have the H3K9 methyltransferase Clr4 and thus H3K9 methylation is absent; hence they are devoid of heterochromatin, since Clr4 is the only methyltransferase in *S. pombe*. Therefore, comparison with wild type cells shows how heterochromatin changes RNA Pol II occupancy. It was shown that at centromeric *dg*/*dh* and subtelomeric *tlh* repeats, RNA Pol II occupancy is increased five-fold (Figure 3.2A) and four-fold (Figure 3.2B) respectively in *clr4*Δ when compared to wild type cells. At the remaining subtelomeric region and *mat* locus, such a strong change in RNA Pol II occupancy in absence of heterochromatin was not observed, indicating that at these regions heterochromatic silencing does not reduce RNA Pol II occupancy.

3.2. Contribution of each pathway to heterochromatic silencing

Results showed that heterochromatic silencing is a combination of reduced RNA Pol II occupancy, reduced transcriptional efficiency and reduced RNA stability, however, contribution of each pathway to silencing remains unclear. To determine the effect of heterochromatic silencing, we compared the three pathways at heterochromatic loci between wild type cells and cells having a deletion of the H3K9 methyltransferase *clr4*. At centromeric and subtelomeric *tlh* repeats, heterochromatic silencing is a combination of reduced RNA Pol II occupancy (Figure 3.1), reduced transcriptional efficiency (Figure 3.2) and increased RNA degradation (Figure 3.3). We observe that in absence of heterochromatin, RNA Pol II is more efficiently transcribing unrepetitive regions. Although RNA Pol II occupancy increased ~4-fold in *clr4Δ* cells when compared to wild type cells, the increase in nascent RNA was ~5-fold for centromeric *dg* repeats (Figure 3.2A) and as much as ~12-fold for subtelomeric *tlh* (Figure 3.2B). These data show that reduced transcriptional efficiency is dependent on heterochromatin, and in absence of heterochromatin these regions have higher transcriptional efficiency that is comparable to euchromatic regions. Notably, these data show that the DNA sequence is not the reason for decreased transcriptional efficiency, but that this reduction is mediated by the heterochromatin.

In *clr4*Δ cells, stability of centromeric *dg* transcripts was increased, showing that heterochromatin targets these RNA molecules to degradation (Figure 3.2A). Although heterochromatin was eliminated from all regions, subtelomeric *tlh* RNAs were still more rapidly degraded than centromeric transcripts in *clr4*Δ cells (Figure 3.2). This is consistent with the previous finding that at the subtelomeric *tlh* region, the Ccr4-Not complex degrades RNA in parallel to heterochromatic silencing (Brönner *et al.*, 2017). In summary, RNA degradation

at centromeric repeats is heterochromatin dependent, whereas at subtelomeric *tlh* repeats multiple pathways degrade heterochromatic RNA.

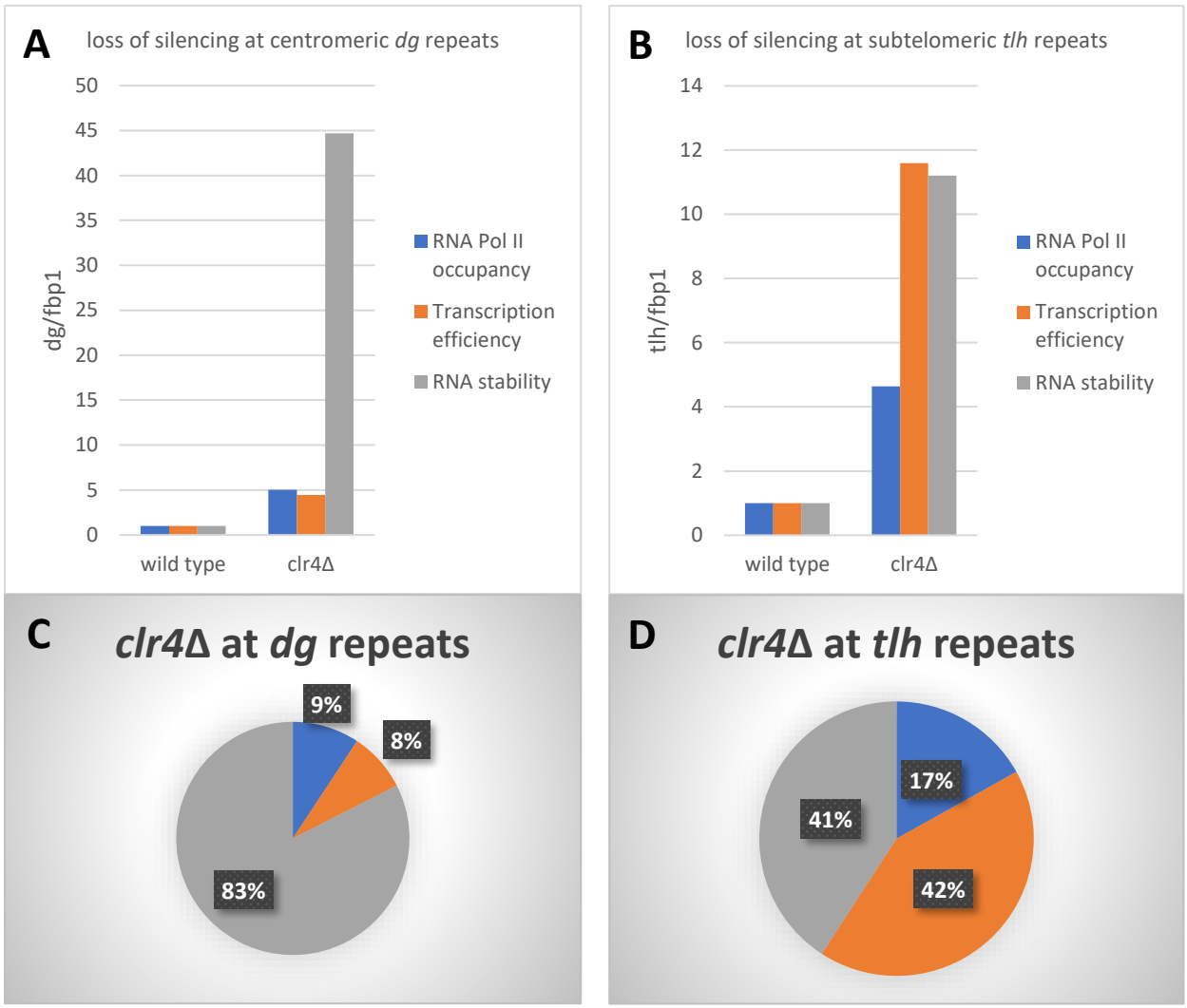

Figure 3.2. Loss of silencing of the three pathways (Pol II occupancy, transcription efficiency and RNA stability) at centromeric *dg* (A) and at subtelomeric *tlh* (B) in *clr4*Δ cells relative to wild type. Pie charts depict the effect of *clr4*Δ in loss of silencing as percentage for centromeric *dg* (C) and subtelomeric *tlh* (D). Figures demonstrate contribution of the three pathways to heterochromatic silencing at repeats in fission yeast. Average of at least two independent samples is shown for all figures.

Next, the relative contribution of each pathway to heterochromatic silencing was quantified, by comparing the data of wild type and *clr4*Δ cells. At centromeric repeats, RNA degradation (83%) contributes to silencing more than transcriptional silencing (17%, RNA Pol II occupancy and transcription efficiency in total) in wild type cells (Figure 3.2C). In the transcriptional silencing pathway, RNA Pol II occupancy (9%) contributes slightly more than transcriptional efficiency (8%) to silencing at centromeric repeats. At subtelomeric *tlh* repeats, heterochromatic silencing is primarily transcriptional (42%), whereas RNA degradation contributes less than transcriptional silencing (Figure 3.2D). At this locus, silencing is predominately mediated through reduced transcriptional efficiency (42%). At the

remaining subtelomeric region and the *mat* locus silencing is also predominately transcriptional, with transcriptional efficiency being the dominant pathway.

3.3. Contribution of the Ccr4-Not complex to distinct silencing pathways

Next, RNA Pol II occupancy, transcriptional efficiency and RNA degradation were analyzed in strains having deletions of the Ccr4-Not complex. The Ccr4-Not complex is not limiting RNA Pol II access at the subtelomeric region (Figure 3.3A). However, it might influence RNA Pol II occupancy at the centromeric heterochromatin (Figure 3.3B). On the other hand, a strong recovery of transcriptional efficiency was observed in the deletion of the Ccr4-Not component *caf1* (Figure 3.3A). This indicates that the deadenylase activity of the Ccr4-Not complex is required for reduced transcriptional efficiency in wild type cells and that the Ccr4-Not complex modulates RNA Pol II activity in a heterochromatin dependent manner. The remaining subtelomeric genes and *mat* locus show similar requirements for reduction in transcriptional efficiency as subtelomeric *tlh* repeats. Upon deletion of *caf1* an increase in RNA degradation was observed, consistent with the increased transcriptional efficiency and higher amounts of nascent RNA in this mutant (Figure 3.3).

Contribution of silencing of the Ccr4-Not complex was quantified by comparing the data of mutant strains to the wild type. According to these data, Caf1 contributes to silencing mainly by reducing the transcription efficiency, both at subtelomeric region (Figure 3.3C) and at the centromeric repeats (Figure 3.3F). On the contrary, Ccr4 mediates silencing through the reduction of RNA Pol II access, primarily at the centromeric repeats (Figure 3.3D) and at a lower level at the subtelomeric region (Figure 3.3G). In the activity mutant, transcription efficiency had a slightly higher effect than the other silencing pathways (Figure 3.3E, 3.3H).

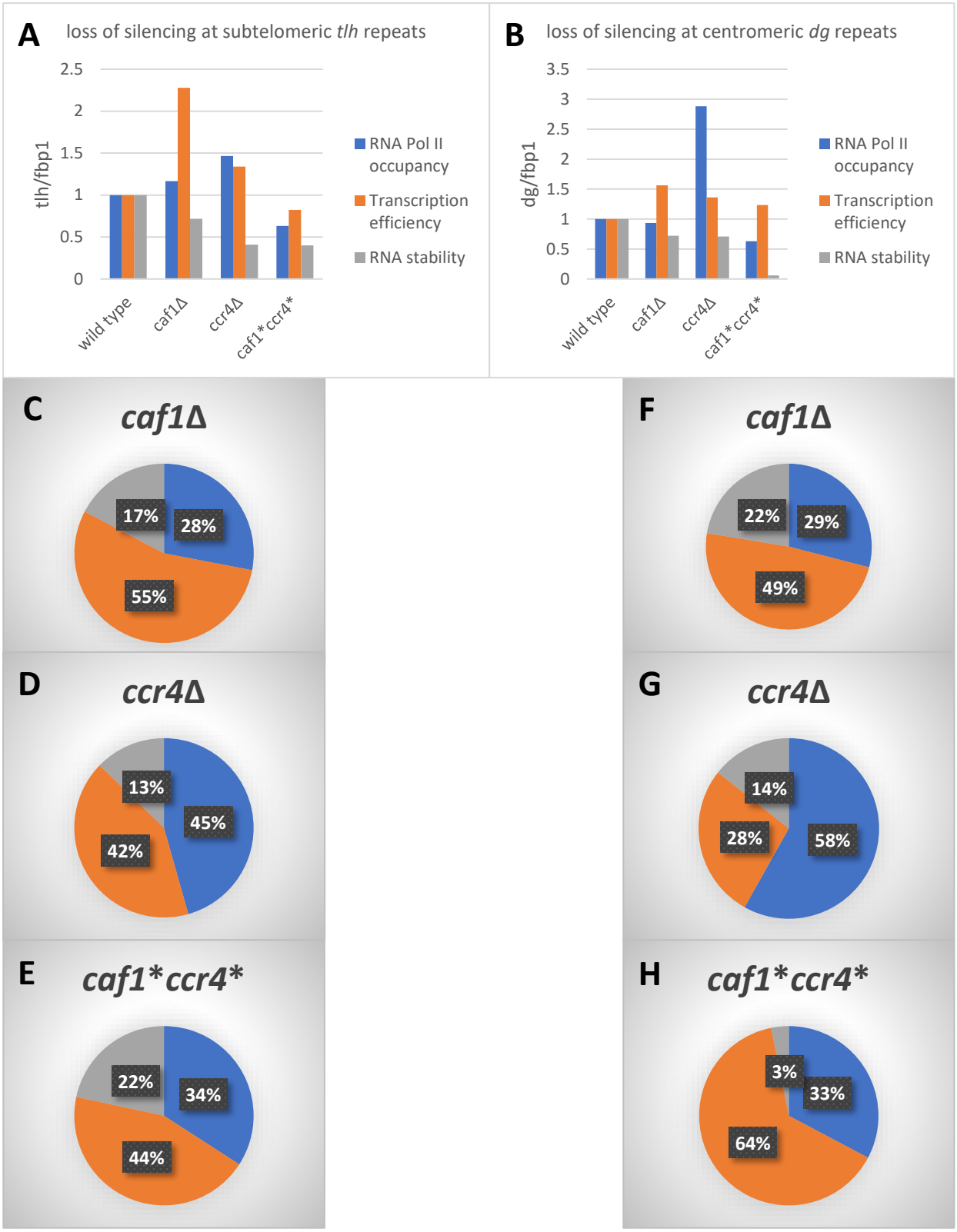

Figure 3.3. Loss of silencing at centromeric and subtelomeric repeats in indicated mutant cells relative to wild type. Loss of silencing is shown for each of the three pathways RNA Pol II occupancy (Pol II ChIP), transcription efficiency (Pol II RIP / Pol II ChIP) and RNA stability (pA RNA / Pol II RIP). Pie charts (from C to H) show the contribution of each pathway to silencing in the given mutant. List of mutants is in Table 2.1.

It was previously shown that the Ccr4-Not complex degrades subtelomeric RNA redundantly with RNAi (Brönner *et al.*, 2017). Current data show that the Ccr4-Not complex is also required for silencing at the transcriptional level, where it specifically regulates RNA Pol II transcription efficiency at subtelomeric *tlh* and centromeric *dg* repeats (Figure 3.3A-H). In agreement with the previous data, deletion of *caf1* had no or little effect on RNA stability and RNA Pol II occupancy, but it is completely essential for reduced transcriptional efficiency.

In summary, results show that heterochromatic silencing acts at different levels. First, RNA Pol II accessibility is reduced at heterochromatic regions, which also reduces overall transcription at these loci. Second, transcriptional efficiency was identified as a new mode of heterochromatic silencing. Although, RNA Pol II is present at heterochromatic loci, the transcriptional efficiency is reduced, which is regulated by heterochromatin and the Ccr4-Not complex. This mode of silencing is present at all heterochromatic regions in fission yeast cells. Third, the final control of heterochromatic silencing is RNA degradation by RNAi and several RNA degradation machineries, including the Ccr4-Not complex.

4. DISCUSSION

4.1. Heterochromatic silencing acts at three different levels

Results of this work indicate that heterochromatic silencing consists of three pathways: reduced RNA Polymerase II occupancy, reduced transcriptional efficiency and reduced RNA stability. In wild type cells RNA Polymerase II occupancy is reduced at several but not all heterochromatic regions in fission yeast cells. It was noted that RNA Polymerase II strongly enriches on chromatin in those mutants which greatly affect H3K9me levels, such as *clr4*Δ at centromeres (Okita *et al.*, 2019). Reduced RNA Polymerase II access has been initially proposed as a mode of heterochromatic silencing and has been observed in many organisms (Feng and Michaels, 2015; Grewal and Elgin, 2007).

In addition to reduced RNA Polymerase II occupancy, reduced transcriptional efficiency was identified as another mode of transcriptional silencing. Although RNA Polymerase II is present at heterochromatic regions, its RNA production (nascent RNA) is lower than in euchromatic regions. This mode of silencing is present at all heterochromatic regions in fission yeast cells, suggesting that it is likely conserved in other organisms as well. This mode of silencing might be analogous to SIR mediated silencing in *S. cerevisiae*. Biochemical data show that in silent chromatin in *S. cerevisiae*, RNA Polymerase II is allowed to initiate transcription, but the SIR complex blocks elongation on chromatin and maintains RNA Polymerase II in a stalled conformation (Johnson *et al.*, 2013).

Heterochromatin is essential to reduce transcriptional efficiency and in absence of H3K9me, transcription efficiency at heterochromatic loci is increased to the level of euchromatic genes. These results also show that reduced transcriptional efficiency is not encoded in the DNA sequence, but it is induced by the heterochromatin structure. Moreover, heterochromatin is crucial for heterochromatic transcript degradation. It was shown that in *clr4*Δ cells, stability of centromeric *dg* transcripts was increased, which means that heterochromatin targets these RNA molecules to degradation. The detailed mechanism of this degradation, however, remains to be elucidated.

4.2. The Ccr4-Not complex has a central function in heterochromatin transcription

Notably, it was revealed that reduced transcriptional efficiency is mediated by the Ccr4-Not complex. In *caf1*Δ cells, RNA Polymerase II occupancy was not changed at heterochromatic regions, however, transcription efficiency was increased to the level of euchromatic genes. H3K9 methylation is not lost in *caf1*Δ cells at subtelomeric *tlh* and centromeric *dg*/*dh* repeats (Brönner *et al.*, 2017; Cotobal *et al.*, 2015; Sugiyama *et al.*, 2016), suggesting that heterochromatin might be functional, however, the transcriptional efficiency is increased to the level of euchromatic genes. These data show that the Ccr4-Not complex regulates transcriptional efficiency post heterochromatin formation. H3K9 methylation and heterochromatin are, however, required for this function of the Ccr4-Not complex. These data indicate, that there is a subset of RNA Polymerase II complexes in heterochromatic regions, which are phosphorylated at the serine residues (S2 phosphorylation), but do not produce RNA molecules. The Ccr4-Not complex reduces transcriptional efficiency of RNA Polymerase II in a heterochromatin dependent manner and might act in an analogue way to the SIR complex in *S. cerevisiae* (Johnson *et al.*, 2013).

The Ccr4-Not complex was initially described as a chromatin associated complex involved in transcription (Miller and Reese, 2012), and new data support more and more this mode of activity. In this study, deletion of caf1 resulted in an increase in RNA degradation, consistent with the increased transcriptional efficiency and higher amounts of nascent transcripts in this mutant. These data are in agreement with the previous finding that RNAi acts in parallel to Ccr4-Not to degrade heterochromatic RNA (Brönner *et al.*, 2017). In the absence of Ccr4-Not an increase in small RNA generation and RNAi activity was observed (Brönner *et al.*, 2017). The Ccr4-Not complex was shown to act as a transcription elongation factor suggested to reactivate arrested RNA Polymerase II (Dutta *et al.*, 2015; Kruk *et al.*, 2011). It is possible that, in presence of heterochromatic marks, H3K9 methylation and histone deacetylation, Ccr4-Not exhibits an opposite role and stalls RNA Polymerase II. Recently, Ccr4-Not was also shown to be required for DNA-damage dependent ubiquitination and degradation of RNA Polymerase II (Jiang *et al.*, 2019), and it is possible that the Ccr4-Not mediated stalling requires RNA Polymerase II ubiquitination. The Ccr4-Not complex is recruited to RNA Polymerase II by the histone chaperone Spt6 (Dronamraju *et al.*, 2018), which was also implicated in heterochromatin formation in fission yeast (Kiely *et al.*, 2011). Increasingly more data show that Ccr4-Not is recruited to chromatin and regulates RNA Pol II transcription.

In summary, it was shown that heterochromatic silencing consists of reduced RNA Polymerase II occupancy, reduced transcriptional efficiency and increased RNA degradation. Each pathway contributes to silencing at varying degrees and several proteins corroborate to maintain this silencing.

4.3. RNA Polymerase II as target molecule for transcriptional analysis

The largest subunit of the RNA polymerase II complex, Rpb1 contains evolutionarily conserved heptapeptide repetitive sequences (Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7) in its C-terminal domain (CTD). The CTD is highly phosphorylated during transcription. The patterns of phosphorylation, glycosylation, isomerization, and the unmodified state of each residue are deciphered to regulate transcription and cotranscriptional events. Increasing number of studies show that these properties of the CTD code can be harvested to obtain insights for the process of transcription at various stages. For example, Ser7P was shown to facilitate heterochromatin formation via non-coding RNAs (Kajitani *et al.*, 2017) and antisense transcription was shown to regulate gene expression in fission yeast (Wery *et al.*, 2018). A similar approach was used in this work where the phosphorylated state of the serine residues of RNA Polymerase II was utilized.

Phosphorylation at Ser5 (Ser5P) is a mark of transcription initiation, and phosphorylation at Ser2 (Ser2P) is a mark of transcription elongation and termination (Hirose and Ohkuma, 2007; Eick and Geyer, 2013). At the beginning of this project, Ser5P was intended to be analyzed in parallel with Ser2P. Unfortunately, the antibody used in ChIP and RIP experiments was shown to bind too weakly to the phosphorylated serine 5 residues and consequently the following sequencing analysis obtained from these experiments were not conclusive. As a result, Ser5P analysis was eliminated from the rest of the work. On the other hand, the antibody against Ser2P showed better binding properties and was used at the rest of the project.

This work and the studies mentioned above point out that the RNA Polymerase II complex holds a great potential for sequencing experiments and is a promising candidate particularly for transcriptomic analyses.

4.4. Future perspectives

Next-generation sequencing technology has revolutionized the research in life sciences. The huge amount of data generated with the help of this application enables to explore the details which were not possible to detect before this technology was developed. At this point, the importance of bioinformatic analysis comes to the fore. Data mining lets the researchers dig out the information which cannot be obtained using conventional tools. Similarly, this work is an example how the usage of data gathered through high-throughput sequencing can give detailed information on a subject of interest.

While the role of Caf1 subunit of the Ccr4-Not complex is better understood, significance of the Ccr4 deadenylase and other subunits of this protein assembly in heterochromatic silencing remains to be studied in future works.

5. CONCLUSION

The Ccr4-Not complex is a major player of a sophisticated network which regulates gene expression. Based on the results of this work, following conclusions can be drawn:

- heterochromatic silencing consists of three pathways: reduced RNA Pol II occupancy, reduced transcriptional efficiency and reduced RNA stability.
- RNA Pol II is strongly enriched on chromatin in those mutants which greatly affect H3K9me levels, such as the *clr4*Δ mutant.
- reduced transcriptional efficiency has been identified as a novel mode of transcriptional silencing.
- reduced transcriptional efficiency is not encoded in the DNA sequence, but it is induced by the heterochromatin structure.
- transcriptional efficiency is mediated by the Ccr4-Not complex.
- the Ccr4-Not complex regulates transcriptional efficiency post heterochromatin formation; H3K9me and heterochromatin are, however, required for this function of the Ccr4-Not complex.
- there is a subset of RNA Pol II complexes in heterochromatic regions, which are S2 phosphorylated, but do not produce RNA.
- the Ccr4-Not complex reduces transcriptional efficiency of RNA Pol II in a heterochromatin dependent manner and might act in an analogue way to the SIR complex in *S. cerevisiae*.

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7. CURRICULUM VITAE

PERSONAL INFORMATION	Haris Amedi
EDUCATION AND TRAINING	
01/10/2015–present	Master's study of Molecular Biology
	Faculty of Natural Science, University of Zagreb, Zagreb (Croatia)
25/09/2008–11/02/2015	Bachelor's study of Molecular Biology
	Faculty of Science, "Ss. Cyril and Methodius" University, Skopje (Republic of North Macedonia)
WORK EXPERIENCE	
15/03/2017–15/06/2018	Erasmus+ Traineeship and Master's thesis
	Gene Center Munich, Ludwig-Maximilians-Universität, Munich (Germany) General - experimental design, effective use of scientific literature, data analysis, interpretation of the experimental results, presentation skills, basic safety rules in a laboratory Occupational - basic methods in molecular biology: DNA & RNA isolation from the fission yeast, PCR, gel electrophoresis, Chromatin Immunoprecipitation (ChIP), RNA Immunoprecipitation (RIP), Sanger sequencing, Next-Generation Sequencing (NGS), cloning of DNA fragments
15/03/2016–30/06/2016	Internship
	Laboratory for Molecular Anthropology, Institute for Anthropological Research, Zagreb (Croatia) - mitochondrial DNA (mtDNA) isolation - PCR - Electrophoresis - Sanger sequencing - Statistical analysis
20/05/2013–20/06/2013	Internship
	Institute for Experimental Medicine, Faculty of Medicine, University of Istanbul, Istanbul (Turkey) - Isolation of DNA & RNA - Quantitative PCR (qRT-PCR) - Cell culture - Restriction Fragment Length Polymorphism (RFLP)
01/11/2012–28/02/2013	Bachelor's Thesis

	Laboratory of Microbiology and Microbial Biotechnology, Faculty of Science, "Ss. Cyril and Methodius" University, Skopje (Republic of North Macedonia) - antimicrobial activity of the extracts of some mushrooms from various forests of Macedonia
10/04/2011–20/05/2011	Volunteer
	Laboratory for Biochemistry, Molecular Biology and GMO Control, Department of Genetics and Selection, Institute for Plant Biotechnology, Faculty of Agricultural Sciences and Food, "Ss. Cyril and Methodius", Skopje (Republic of North Macedonia) - application of molecular markers for genotyping and selection of plants
PERSONAL SKILLS	
Mother tongue(s)	Turkish
Other language(s)	Croatian, Macedonian, English
Communication skills	・good communication skills gained through a private business in Croatia ・excellent contact skills acquired during my master's thesis in Germany in a very international environment ・attention, awareness and teamwork skills ・sense of humor
Digital skills	- good command of Microsoft Office applications - design skills gained from personal computer
Other skills	music: former guitarist of a rock band (good at classical, acoustic and electric guitar) sports: basketball, football, jogging, swimming, karate (brown belt)