Role of Transcription Termination Factors Rtt103-Rail-Ratl in Gene Regulation

Thesis submitted for the degree of

Doctor of Philosophy

work carried out by

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DECLARATION

I, **Kathirvel Ramalingam**, hereby declare that this thesis entitled **"Role of transcription termination factors Rtt103-Rai1-Rat1 in gene regulation"** submitted by me under the guidance and supervision of **Prof. Krishnaveni Mishra**, is an original and independent piece of research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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This is to certify that the thesis entitled **"Role of transcription termination factors Rtt103-Rai1-Rat1 in gene regulation"** submitted by **Kathirvel Ramalingam** bearing registration number **17LBPH03** in partial fulfilment of the requirements for the award of Doctor of Philosophy in the School of Life Sciences is a bonafide work carried out by him under my supervision and guidance.

The thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma.

The student has the following publications prior to submission:

- **Ramalingam, K.** & Mishra, K. Transcription termination complex, Rtt103-Rai1-Rat1, regulates sub-telomeric transcripts in Saccharomyces cerevisiae. *RNA Biol.* **20**, 95–108 (2023).

- Abraham, N. M., **Ramalingam, K.**, Murthy, S. & Mishra, K. Siz2 Prevents Ribosomal DNA Recombination by Modulating Levels of Tof2 in *Saccharomyces cerevisiae*. *mSphere* **4**, (2019).

Part of this work has been presented at the following conferences:

- "Yeast India-2023", March 2023, IISER, Mohali, India.
- "Yeast Genetics Meeting-2022", August 2022, University of California, Los Angeles, USA.
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- "20th-Transcription Assembly Meeting-2018"- May 2018, CDFD, Hyderabad, India.
- Indo-US conference on 'Transcription, Chromatin Structure DNA Repair and Genomic Instability', March 2018, IISc, Bangalore.

Further the student has passed the following courses towards fulfilment of coursework requirement for Ph.D.

Course code	Name	Credits	Pass/Fail
BC 801	Research Methodology/Analytical techniques	4	Pass
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BC 803	Scientific Writing and Research Proposal	4	Pass

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So now let me take you through a journey of mystery, enigmas and questions waiting to be solved...

LIST OF ABBREVIATIONS USED

5-FOA	5-Fluoroorotic Acid
Afs	Assembly factors
ANOVA	Analysis of Variance
BFR1	BreFeldin A Resistance
CEN	Centromere
CF	Cleavage Factor
ChIP	Chromatin Immunoprecipitation
CHX	Cycloheximide
CID	CTD Interacting Domain
COMPASS	Complex of Proteins Associated with Set1
CPF	Cleavage and Polyadenylation Factor
CTD	Carboxy Terminal Domain
CUTs	Cryptic Unstable Transcripts
DDR	DNA damage response
DNA	Deoxyribonucleic Acid
DSB	Double Strand Break
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FACT	Facilitates Chromatin Transcription
FDR	False Discovery Rate
GFP	Green Fluorescent Protein
GTFs	General Transcription Factors
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HR	Homologous Recombination
HRP	Horse-Radish Peroxidase
IP	Immunoprecipitation
K-H	Kub-5 Hera

KO	Knock-Outs
LB	Luria-Bertani Broth
LiCl	Lithium Chloride
M	Molar
MAT	Mating Type
miRNA	microRNA
ml	millilitre
mM	Millimolar
MMS	Methyl Methane Sulfonate
mRNA	messenger RNA
mRNP	messenger Ribonucleoprotein
ncRNA	non-coding RNA
NELF	Negative Elongation Factor
NHEJ	Non-Homologous End Joining
NLS	Nuclear Localization Signal
NMR	Nuclear Magnetic Resonance
OD	Optical Density
PAF1	Polymerase associated factor 1
PAGE	Polyacrylamide Gel Electrophoresis
PCI	Phenol-Chloroform-Isoamyl alcohol
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PIC	Pre-Initiation Complex
PVDF	Polyvinylidene fluoride
qRT-PCR	quantitative Reverse Transcription PCR
RAI1	Rat1p Interacting protein 1
RAT1	Ribonucleic Acid Trafficking
RNA	Ribonucleic Acid
RNA Pol I	RNA Polymerase I

RNA Pol II	RNA Polymerase II
RNA Pol III	RNA Polymerase III
RNPs	Ribonucleoprotein Particles
RPM	Revolutions Per Minute
RPs	Ribosomal Proteins
rRNA	ribosomal RNA
RTT103	Regulator of Ty1 Transposition
SC	Synthetic Complete
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of the Mean
SGA	Synthetic Genetic Array
SGD	Saccharomyces Genome Database
snoRNA	small nucleolar RNA
snRNA	small nuclear RNA
ssDNA	Single Stranded DNA
STREs	Stress-Responsive Elements
SUTs	Stable Uncharacterized Transcripts
TAS	Telomere-Associated Sequences
TBS	Tris-Buffered Saline
TCA	Trichloroacetic acid
TERRA	Telomeric repeat–containing RNA
TPE	Telomere Position Effect
tRNA	transfer RNA
ts	temperature-sensitive
TSR4	Twenty S rRNA accumulation
UTR	Untranslated Region
UV	Ultraviolet
WT	Wild Type
YPD	Yeast extract-Peptone-Dextrose

YPK	Yeast extract-Peptone-Potassium Acetate
µl	microlitre

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CHAPTER 1

Introduction

In eukaryotes, the regulation of gene expression is primarily determined by transcription, which is crucial for cellular development and homeostasis. The cells employ three functionally and structurally related RNA polymerases to amplify a subset of transcripts selectively[1]. RNA polymerase I (RNA Pol I) amplifies the highly abundant ribosomal RNAs (rRNAs), whereas RNA polymerase III (RNA Pol III) is dedicated for the amplification of transfer RNAs (tRNAs), 5S-rRNA, and U6-spliceosomal snRNA. All protein-coding genes and several other non-coding RNAs (snRNAs, snoRNAs, miRNAs, SUTs, and CUTs) are transcribed by a single highly conserved enzyme RNA polymerase II (RNA Pol II).

As transcript levels are significant regulators of protein levels within the cell, the regulation of transcription and proper production of matured transcripts is critical in determining cellular growth and survival[2]. Furthermore, transcriptional misregulation is associated with various human diseases and disorders[3]. In a densely packed genome like that of *Saccharomyces cerevisiae,* transcription by RNA pol II is a stringent and highly regulated process governed by multiple factors. These include various general transcription factors, chromatin modifiers, activators, repressors, and RNA processing elements[4]. Despite several years of research, how all these factors work together in concert to regulate transcription is not entirely understood. Most of our current knowledge regarding transcriptional regulatory mechanisms is based on the studies done using *Saccharomyces cerevisiae.* These studies indicate that there is a remarkable amount of conservation that exists between yeast and humans in the molecular mechanisms that underlie transcription by RNAPII.

1.1 Transcription cycle

In eukaryotes, the transcription cycle is broadly classified into three phases: initiation, elongation, and termination. It is co-transcriptionally accompanied by three RNA processing events: capping, splicing, and polyadenylation. Additionally, the RNA exosome complex also associates co-transcriptionally and is involved in the regulation of 3′-end processing and

suppression of transcription read-through[5]. A key event that coordinates the phases of transcription is the phosphorylation status of the carboxy-terminal domain (CTD) of the largest subunit, Rpb1[6]. RNA pol II CTD consists of an extended hexapeptide tail repeats Tyr^1-Ser^2-Pro^3-Thr^4-Ser^5-Pro^6-Ser^7, where Tyr^1, Ser^2, Thr^4, Ser^5, and Ser^7 residues can be phosphorylated. This repeat, which is a unique feature of RNA Pol II, is conserved across all eukaryotic Pol II molecules. The differential and reversible phosphorylation status within the consensus sequence facilitates the recruitment of factors and complexes required for each stage of transcription (Figure 1.1). In transcription, the initiation and elongation steps have been better understood compared to the termination event. As the work reported in the thesis is focused on the factors involved in transcription termination, this chapter provides an overview of the general transcription cycle and transcription termination of protein-encoding genes, with a particular focus on the transcription termination complex (Rtt103-Rai1-Rat1).

Initiation

Briefly, the first step in initiation involves the recruitment of RNA Pol II to the promoter and melting of DNA to expose the template strand. This is aided by general transcription factors (GTFs) and the mega-dalton mediator complex, constituting the stable pre-initiation complex (PIC)[4,7]. The primary function of the mediator complex is to transmit information converging from GTFs directly onto the CTD of RNA Pol II. Depending on the developmental and environmental cues, different GTFs associate with different mediator sub-units and facilitate regulated gene expression[8]. The access to move further is provided by auxiliary factors, which regulate chromatin structure (ATP-dependent chromatin remodellers, histone acetylases, and histone methylases). Similarly, the transition of PIC to a stable initiation complex and its subsequent transition to an elongation-competent complex is facilitated by CTD of RNA Pol II[9]. Once the transcription apparatus leaves the promoter, the PIC is not entirely disassociated, leaving behind a subset of transcription factors termed scaffold-complex, which initiates the next round of transcription[10].

The transcription apparatus undergoes several rounds of abortive initiation before it forms a stable 8-9 nucleotide DNA-RNA hybrid to enter the productive elongation phase[11]. Subsequently, the transcription machinery is subjected to checkpoint control at promoter-

proximal sites by Spt4/5 and NELF (Negative elongation factor) for 5′ mRNA capping[12]. During this window, the Ser[5] phosphorylation of the CTD dominates and recruits the capping machinery. Shortly after capping, the repressive nature of the Pol II-Spt4/5 complex is reversed by cyclin-dependent kinase Ctk1/Bur1[13,14]. It selectively phosphorylates Spt5 and Rpb1-CTD at Tyr[1] and Ser[2] respectively, which aids the transcription machinery to proceed into the productive elongation phase[15]. Structural studies suggest that Spt4/5 complex binds directly to RNA Pol II and improves the processivity of RNA polymerase by preventing its dissociation from the template[16].

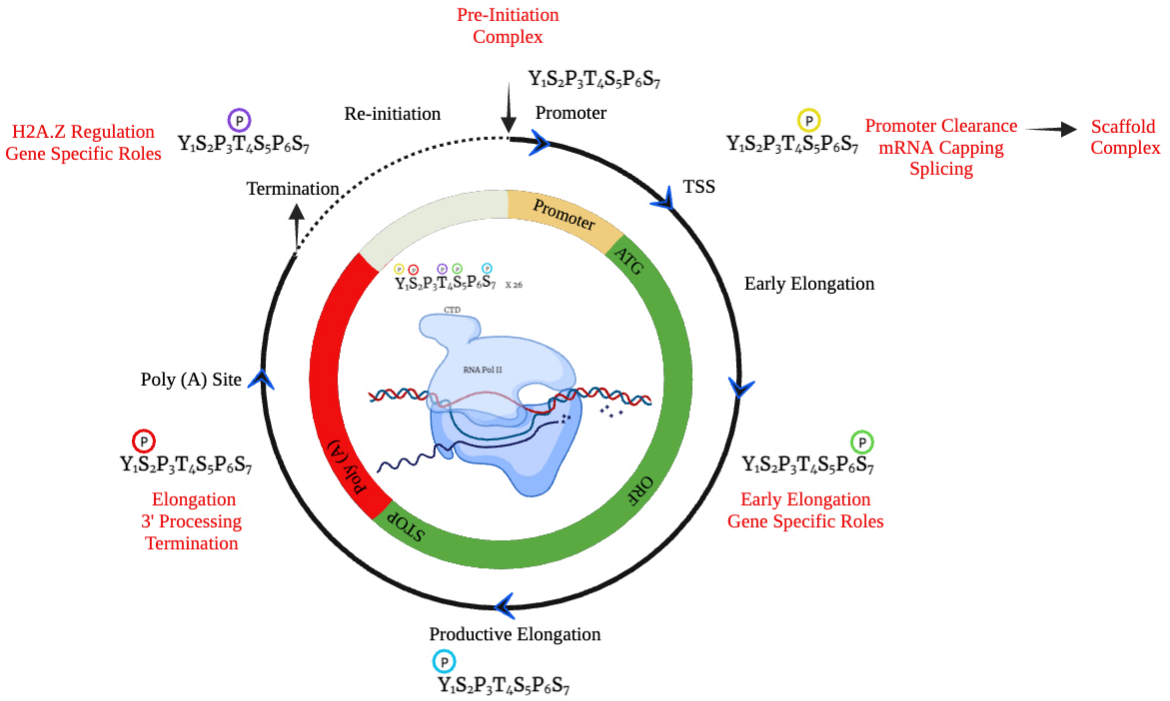

Figure 1.1: The transcription cycle: The major events of the transcription cycle and its accompanying CTD phosphorylation events of the RNA Pol II are depicted in this figure. The inner circle illustrates the occurrence of events in relation to the gene body. The transition of hypo-phosphorylated RNA Pol II from the promoter and its major dynamic phosphorylation status on the hexapeptide repeat is indicated by circled P above the residue. The phospho-CTD-associated process is depicted in red.

Elongation

The transcription machinery has to contend with different obstacles on its way along the gene. These include highly condensed heterochromatin structures, drug-induced roadblocks, sequence-dependent pause, and replicating DNA polymerase complex. The dominance of Ser^2 phosphorylation on the CTD tail and Ser^2P facilitated recruitment of accessory factors like Set1 (histone modifying enzyme)[17]; Spt6 and FACT (Facilitates Chromatin Transcription) (histone chaperones)[18,19]; and RSC[20], Chd1[21,22], and ISW1(chromatin remodeling complexes)[23] marks the successful elongation and aids the smooth transition of transcription machinery through the gene body. The C-terminal region of Spt5 recruits the multifunctional polymerase-associated factor 1 (Paf1) complex[13] (Paf1, Leo1, Ctr9, Cdc73, and Rtf1), which in turn acts as a platform to recruit Set1 (H3K4 methyl transferase), a subunit of COMPASS (Complex of Proteins Associated with Set1), which catalyzes di and tri-methylation of H3K4 on chromatin templates[24]. Together FACT and Spt6 histone chaperones facilitate the disassembly and assembly of nucleosomes for the transcription machinery to transverse through the gene body. Besides, the presence of A/T rich or unusual bases often results in backtracking of RNA Pol II, causing misalignment of the active site with the growing 3′-end of nascent mRNA. The elongation factor -TFIIS, acts on the back-trapped polymerase and stimulates its intrinsic weak 3′→ 5′ exoribonuclease activity for the extrusion of misaligned bases and re-aligns the RNA pol II active site[25,26].

Termination

Once the RNA Pol II reaches the 3′-end of the genes, the elongation complex transits itself into the final stage termed as termination. Termination involves the processing of nascent transcript and the release of RNA Pol II from the template. An appropriate termination event is critical as it prevents transcriptional interference with the DNA downstream and promotes efficient polymerase recycling[27,28]. Furthermore, it stabilizes the nascent mRNA by polyadenylation and aids the recruitment of ribonucleoprotein particles (RNPs) to facilitate the export of mRNAs[29,30]. The termination of RNA Pol II does not occur at a constant distance or confined to a specific region at the 3′-end, rather it terminates from a few nucleotides to several kb downstream of the poly (A) site and appears random[31]. It is still one of the least understood processes and is governed by multiple cis- and trans-acting factors.

With the in-depth analysis of transcription machinery over the years, it is now being revealed that the termination is executed via two different mechanisms[30,32–34]: the poly(A)-dependent pathway for protein-coding genes executed by the Rtt103-Rai1-Rat1 complex and the Sen1-dependent pathway executed by Nrd1-Nab3-Sen1 complex, which preferentially terminates non-coding RNA and short coding transcripts. The specificity of a particular pathway is typically determined by a combination of termination signals present on the nascent RNA molecule and the recognition of specific phosphorylation patterns on the carboxy-terminal domain (CTD) of the largest subunit of RNA Pol II. The choice of termination pathway has a decisive influence on the fate of mRNA[32,35]. However, many recent global genomic studies have suggested that there may not be a strict distinction between the two pathways in terms of the association of termination factors between protein-coding vs non-coding genes and some cross-talk may exist. The ability of the organism to switch between the termination pathways suggests that there are many uncovered functional commonalities that exist between the two termination pathways[29,32,36].

1.2 Transcription termination of protein-coding genes

In most protein-coding genes, the disassociation of RNA Pol II occurs downstream of an RNA maturation event in which the 3′-end of the nascent transcript is cleaved and polyadenylated[37,38]. Based on the mechanistic feature, two models have been proposed for termination, i.e., the allosteric and torpedo models[30,32,34] (Figure 1.2). The allosteric model postulates conformational changes in RNA Pol II due to the loss of elongation factors or anti-termination factors while transcribing past the poly(A) region resulting in termination[39]. Whereas the torpedo model proposes that the 3′-end cleavage of the growing nascent mRNA by CPF (cleavage and polyadenylation factor) and CF (cleavage factor) complex provides an entry point for Rtt103-Rai1-Rat1 complex, where Rat1 via its 5′-3′ exonuclease catches up the transcribing RNA Pol II and dismantles its association with the template[31,40]. Although both models have been supported by considerable experimental evidence, neither of them on its own

is sufficient to explain the process of termination. Hence it has been proposed that the process of termination might occur via a unified allosteric-torpedo model[29,34,41,42] .

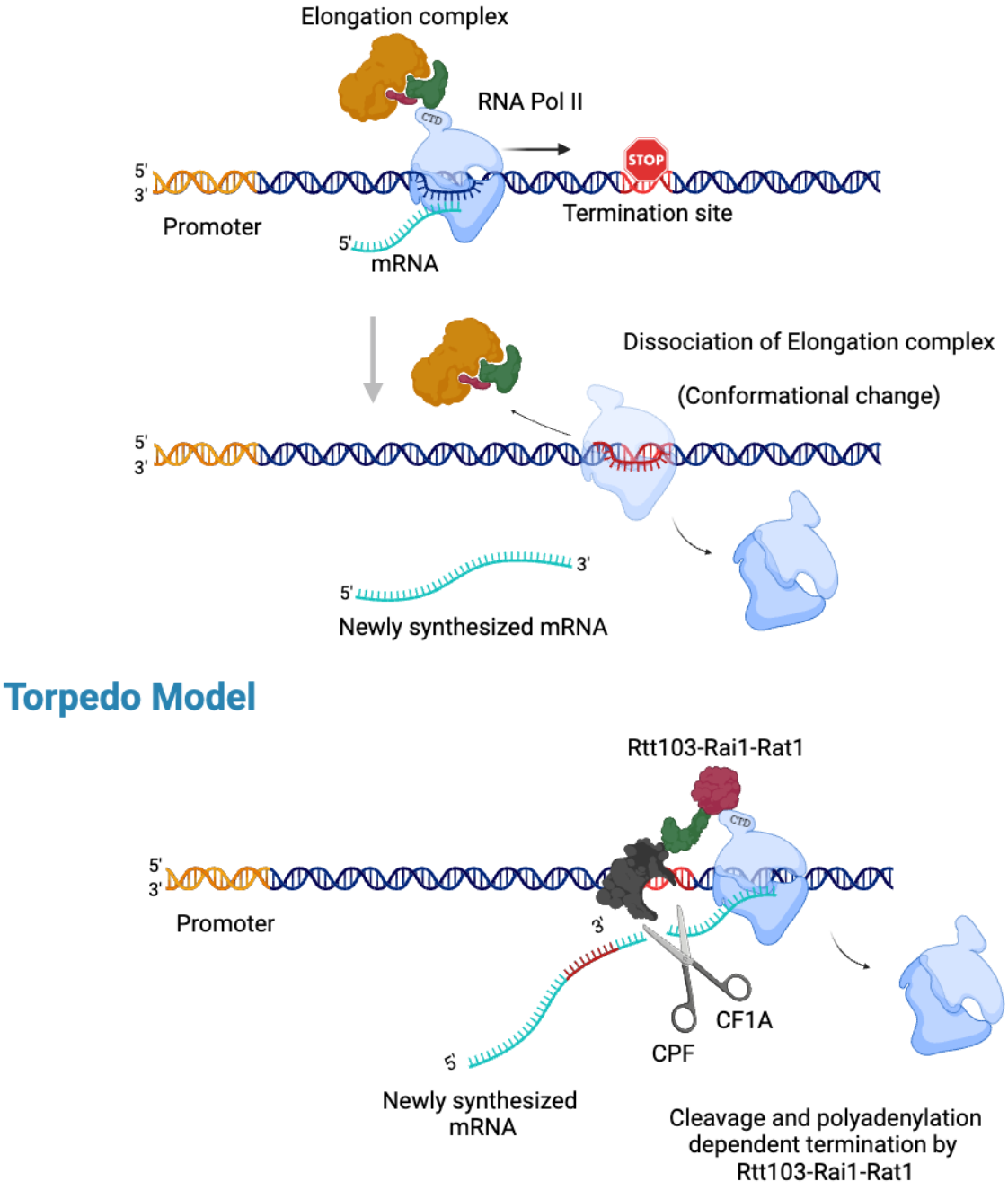

Figure 1.2: Transcription termination of protein-coding genes: The allosteric model – once the transcription machinery surpasses the poly (A) site, loss of either elongation complex or anti-termination factors results in a conformational change on RNA Pol II leading to its dissociation.

The torpedo model – the growing nascent transcript is cleaved by CPF (cleavage and polyadenylation factor) and CF (cleavage factor) downstream of the poly (A) site, which provides an entry point for Rtt103-Rai1-Rat1 complex to co-transcriptionally degrade downstream RNA product and dismantle the transcribing machinery.

1.3 Rtt103

Rtt103 is a highly abundant nuclear protein conserved across all the eukaryotes[43]. In yeast, it was initially isolated in a screen for mutants, which exhibited exacerbated levels of Ty1 element transposition (*RTT103* – Regulator of Ty1 Transposition)[44]. *rtt103*Δ cells displayed approximately 13-fold increase in the mobility of Ty1 elements. Sequence analysis of Rtt103 revealed that it contains an RPR domain (also known as C-terminal interacting domain or CID for specifically interacting with the CTD of RNA polymerase II), which is present in several proteins involved in the regulation of nuclear pre-mRNAs, and facilitates interaction with the C-terminal domain of RNA PolII[45] (Figure 1.3). In yeast, Pcf11 and Nrd1 are the only two other proteins that possess a CID domain. Pcf11 and Nrd1 are involved in the 3′-end processing of mRNA and small nucleolar RNA (snoRNA), respectively, via interaction with RNA Pol II CTD domain when it is phosphorylated at Ser^2 position of the hexapeptide tail (Tyr^1-**Ser^2**-Pro^3-Thr^4-Ser^5-Pro^6-Ser^7)[46].

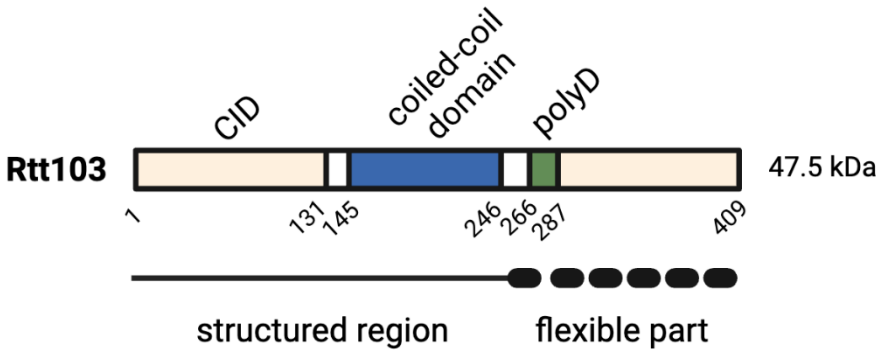

Figure 1.3: Schematic representation of Rtt103 domain organization: The numbers represent the borders of amino acid segments. Structured and flexible regions have been determined based on a limited proteolysis study[45].

The first line of evidence for Rtt103 as a transcription termination factor was documented by Kim *et al.,* in 2004[47]. They found that Rtt103 co-purifies with serine 2 phosphorylated CTD via affinity chromatography. Tandem affinity purification of Rtt103 revealed its association with RNA Pol II, Rai1, and Rat1. Further, ChIP experiments suggested that Rtt103, along with Rai1 and Rat1, crosslinks at the 3′-end of the genes. Rat1 is an essential protein with 5′-3′ exoribonuclease activity, whereas Rtt103 and Rai1 are dispensable for growth under optimal conditions. In a *ctk1*Δ mutant, Rtt103 failed to crosslink at the 3′-end of the genes, suggesting that the Ctk1 mediated phosphorylation of RNA Pol II at the ser^2 position of the CTD hexapeptide is critical for its recruitment[47].

Recent evidence suggests that Rtt103 also interacts with Thr^4 phosphorylated CTD of RNA pol II in a nearly identical manner as that of Ser^2 [48]. Further genomic, proteomic, and NMR structural studies suggest that Ser^2 and Thr^4 enable the recruitment of Rtt103 to a different class of genes, such as protein-coding and non-coding snoRNA genes. The structural model of the Rtt103–CTD demonstrates that Rtt103 forms dimers and decorates along the length of CTD, like beads on a string and governs the exposure of the CTD sequence to other protein-binding factors[45]. Apart from the above-mentioned reports, numerous single and genome-wide studies have validated the association of Rtt103-Rai1-Rat1 complex at the 3′-end of the protein-coding genes[27,47,49,50].

1.4 The "Torpedo" mechanism of termination and role of Rtt103-Rai1-Rat1

Once the RNA pol II transcribes past the poly (A) region, the CPF and CF complex cleave the growing nascent RNA chain and exposes a naked 5′-monophosphate still associated with the elongating RNA polymerase. It was postulated that Rtt103 interacts with the Ser^2 phosphorylated RNA Pol II and recruits Rai1 and Rat1 to the 3′-end of the genes. Rat1, with its 5′-3′ exonuclease activity degrades the 5′-monophosphate exposed RNA to catch up with the RNA Pol II and destabilizes the interaction of the RNA polymerase with the template, resulting in termination[47,50]. Rat1 has a cytoplasmic counterpart, Xrn1, which has similar exonuclease activity[51]. Both the exonucleases specifically degrade RNA with a 5′-

monophosphate group and single-stranded DNA (ssDNA), whereas they cannot act on RNAs with a 7mG cap, 5′-triphosphate, or 5′-hydroxyl[52]. Both proteins can functionally complement each other when their localization is altered via the addition or deletion of nuclear localization signal (NLS)[53]. Initial purification studies of Rat1 revealed Rai1 (Rat1p interacting protein 1) as its strongest interactor; it enhances the exonuclease activity of Rat1, both in vivo and in vitro[54]. Null mutants of Rai1 and the temperature-sensitive allele of Rat1 display substantial termination defects and stabilization of 3′-UTR transcripts downstream of the poly (A) site[47,50]. These data suggest that in the absence of this complex, the extended 3′-UTR is not degraded.

The mechanistic details of the action of the Rtt103-Rai1-Rat1 complex in transcription termination are still not understood due to the lack of a standard in vitro biochemical assay for validation. Some studies have suggested that Rat1/Rai1 exonuclease activity is insufficient for termination[55] and various other unidentified players and parameters like length of the RNA and pausing of RNA Pol II are critical determinants of proper termination[56,57]. Further, in an in vitro assay, it was shown that while Rat1-Rai1 and Rat1-Rai1-Rtt103 complex could efficiently degrade the RNA with the 5′-monophosphate, RNA polymerase remained competent for the elongation process and no termination or dislodging of the RNA polymerase was observed. Further, this work also showed that the overexpression of Rtt103 was sufficient to rescue termination defects posed by catalytically deficient Rat1[57]. In addition, it has been shown that Rat1/Rai1 complex is also required for efficient termination of transcription by RNA Pol I, where Rnt1 endonuclease cleaves nascent rRNA to create an entry point for Rat1-assisted termination[58]. Apart from the above mentioned studies, several other genome-wide chromatin immuno-precipitation analyses suggest a strong correlation for Rat1 association at the 3′-end of non-coding RNA genes too[59]. It is speculated that Rat1 may work in coordination with Sen1, the helicase involved in the termination of non-coding transcripts because the *rat1-1* and *sen1-1* double mutants exhibited a much more pronounced termination defect in comparison with that shown by either of the single mutants for the genes assessed[60].

In addition to its association with the 3′-end of the genes, both Rat1 and Rai1 were also reported to crosslink with the 5′-end of the genes[27,47]. Whether this is due to promoter-terminator cross-talk via gene looping or a quality control mechanism for assessing 5′-7mG capping awaits further validation. A substantial evidence also suggests that Rat1 influences the elongation rate of RNA Pol II and is involved in the co-transcriptional splicing of introns[61–63]. Additionally, it has been demonstrated that Rat1/Rai1 is also involved in the establishment of

heterochromatin formation and processing of rRNA transcripts[64–66]. These observations clearly suggest that this complex might play multiple roles in the cell beyond its role in transcription termination.

1.5 Termination factors are associated with several disease states

Understanding the contribution of transcription termination factors to gene regulation is important. Work from several laboratories suggests a significant link between transcription termination factors, genome stability, and disease states. In mammals, polymorphisms in Xrn2, the Rat1 homologue, are associated with spontaneous lung carcinoma in non-smokers[67]. PSF/p54nrb, the recruiter of Xrn2, is critical for cellular survival in patients with colon and prostrate cancers[68,69]. Loss of either PSF or p54nrb impaired the repair kinetics of the cell and resulted in increased chromosomal aberrations[70,71]. p54(nrb) is highly expressed and required for the development and progression of malignant melanoma[72]. Sen1, a putative DNA:RNA helicase, which terminates transcription of short-length genes, prevents R–loop mediated genome instability during transcriptional pausing[73,74]. Loss of Sen1 activity is found to be associated with several neurological pathologies[75,76].

1.6 Rtt103 and its role in genome stability

Among the three termination factors of the Rat1 complex, Rtt103 is the least understood. The human homolog of Rtt103 is Kub-5 Hera (K-H)/RPRD1A/CREPT[43]. It is a Ku70 binding protein which mediates the expression of several cell cycle-related genes[77]. Overexpression of K-H in humans resulted in increased rates of cell proliferation and tumorigenesis[77]. A homozygous knock-out of k-h is lethal, whereas the presence of only one allele results in increased R-loop formation and chromosomal aberrations[78]. Overexpression also rescued R–loop mediated genome instability. In humans, K-H also forms additional complexes with its CTD domain and participates in the stabilization of the DNA-repair factor Artemis2[78]. In mammals, besides the already known interacting factors, including Ku70, RNA Pol II, and p15RS, it also associates with proteins involved in RNA metabolism

(Topoisomerase 1 and RNA helicases), DNA repair/replication processes (PARP1, MSH2, DNA-PKcs, MCM proteins, PCNA, and DNA Pol δ), and some proteins involved in metabolic processes, including translation[79].

In yeast, synthetic genetic array (SGA) analysis of *rtt103*Δ mutants with a set of viable deletion collections revealed many synthetic interactions. Synthetic lethality was observed when combined with *CTK1* (Ser^2 Kinase of RNA Pol II), *RAI1* (Transcription termination factor), and *REF2* (Involved in snoRNA and mRNA 3′-end formation). Synthetic slow growth was observed with *CTK2*, several transcription factors involved in elongation or 3' end processing like *SPT4* (The Elongator complex(ELP1- 6), members of the Paf complex, *BUR2*, *HTZ1* and its assembly complex SWR-C, and several ubiquitin/proteasome-related proteins[47,80].

1.7 Scope and aim of the current study

Previous investigations from our laboratory have identified that the loss of Rtt103 in *Saccharomyces cerevisiae* results in sensitivity to various DNA-damaging agents[81]. Overexpression of Rtt103 suppresses temperature and MMS sensitivity of *yku70*Δ, while the double mutants have exacerbated sensitivity to DNA damage. Following our work, the human homologue of Rtt103, Kub-5 Hera, was also shown to interact with Ku70 and loss of Kub-5 Hera leads to genome instability and reduced repair capacity in cell lines[78]. Yku70 is a well-conserved protein with a critical role in non-homologous end joining (NHEJ), telomere homeostasis, and gene silencing. In this thesis, we extended our study to assess whether Rtt103 plays a role in telomere metabolism and elucidate its molecular role in maintaining genome stability. In the course of this investigation, we discovered that *rtt103*Δ is cold-sensitive and therefore attempted to uncover the molecular basis of this phenotype. The three major objectives of this work are as follows:

1. **Assess the role of Rtt103 in sub-telomeric silencing.**
2. **Elucidate the molecular basis of Rtt103 function in genome stability.**
3. **Functional characterization of the cold sensitivity of *rtt103*Δ.**

CHAPTER 2

2.1 Materials and Methods

Construction of yeast strains

The *Saccharomyces cerevisiae* strains used in this study are derivatives of W303 or BY4741 and are listed in Table 2.1. Strains were grown under standard conditions in YPD (Yeast extract, Peptone, Dextrose) or synthetic complete (SC) medium at 28°C. For temperature-sensitive (*ts*) mutants, further specifications are mentioned in methods wherever applicable. Standard procedures were followed for yeast manipulations[82].

Gene disruption or gene tagging was performed by PCR-mediated homologous recombination as described by Longtine *et al*., 1998[83]. Typically, 50 bp of target-specific homologous sequences were added to both forward and reverse primers to target the PCR product to a specific site of integration. Positive transformants were confirmed by colony PCR for correct integration events and western blot analysis with antibodies against the epitope tag of the tagged protein. For knock-outs (KO), known phenotypes were tested.

For micromanipulation, haploid parent strains of the opposite mating type (MAT-a and MAT-α) were crossed on YPD in the form of a cross (**X**) with a significant intersection between them. The plates were then incubated overnight at 28°C. The cells from the intersection were then patched on a selective auxotrophic medium, which only permitted the growth of diploids. The diploids were then transferred onto a low-nutrient medium to induce sporulation. A 1 x 1cm patch was made on a YPK (1% yeast extract, 2% peptone, 2.5% potassium acetate, and 2% agar) plate and incubated at room temperature for 3-5 days. Once the cells underwent the process of sporulation, resulting in tetrads (ascospores) encased in the ascus, asci were subjected to enzymatic lysis with zymolyase 100T (2.5mg/ml) for approximately 1 min 10 s at 30°C. The reaction was stopped by diluting the spores in 1 ml of ice-cold double distilled water. The digested tetrads (50 µl) were then transferred onto agar plates (Difco agar) in a vertical line at the centre, and the tetrads were pulled apart for individual spores using a micromanipulator Olympus BX41.

Storage and maintenance of strains

For routine use, the cultures were streaked for single colonies on YPD or SC (as appropriate) and stored at 4°C. Storage of strains for an extended period of time was done at -80°C in 15% glycerol. One millilitre of overnight grown culture was resuspended in an equal volume of sterile 30% glycerol in cryovials and stored at -80°C.

Yeast spot growth assay

For spotting assays, yeast cells were grown overnight at 28°C in appropriate selection media. Cells were harvested and normalized to 1 OD_{600}. The cultures were 10-fold serially diluted, and 5 µl was spotted onto appropriate agar plates. Plates were then incubated for 2–3 days at 28°C and photographed. For cold sensitivity assays, plates were incubated at 16°C for 6–8 days.

Telomere silencing assay: The 5-FOA concentration used was 1 mg/ml. For the *ADE2* colour-based silencing assay, cells were directly plated on YPD agar plates, incubated at 30°C for 2–3 days, followed by 4ºC for a couple of days for the colour to develop, and then photographed.

Chromatin immunoprecipitation (ChIP)

A single colony of yeast cells was grown overnight and sub-cultured to OD_{600} 0.8-1.0. The cells were cross-linked with formaldehyde (final conc. 1.2%) for 10 minutes and quenched with glycine (360 mM) for 15 minutes. Cells were pelleted and washed twice with ice-cold 1X TBS, resuspended in lysis buffer (50mM HEPES/KOH pH-7.5, 1mM EDTA, 140mM NaCl, 0.1% deoxycholic acid, 1% Triton X-100, 1x-protease inhibitor cocktail) and lysed using 0.5 mm glass beads in a vortex mixer for 20 minutes at 4°C. The chromatin lysate was recovered and sheared by sonicating using 10 sec ON/OFF (Henderson Biomedical MSE/ Amplitude 10) for 5 cycles. The sonicated samples were pre-cleared by spinning at 13000 rpm and 10% of the extract was saved as input for normalisation. IgG Sepharose beads (80 µl bed volume per sample) were washed with lysis buffer and IP was performed overnight at 4°C for protein-A tagged samples. After overnight incubation, the beads were washed once with lysis buffer followed by once each with lysis buffer P500 (50 mM HEPES/KOH pH-7.5, 1 mM EDTA, 500 mM NaCl, 0.1% deoxycholic acid, 1% Triton X-100), then LiCl detergent buffer (10 mM Tris-Cl pH-8, 250 mM LiCl, 1 mM EDTA, 0.5% NP-50, 0.5% deoxycholic acid) and finally twice

with 1X TBS. All the washes were done with 1 ml of respective buffer each with a brief incubation time of 5 minutes at 4°C and centrifuged at 2000 rpm for 3 minutes. The bead-bound chromatin was eluted in 100 µl (1%SDS/1X TBS) + 150 µl (0.67%SDS/1X TBS) for 10 minutes at 65°C. For reversing the cross-link, input and IP samples were treated overnight at 65°C with proteinase K and RNase A. The DNA from bound (IP) and unbound fraction (input) was extracted using an equal volume of phenol-chloroform-isoamyl alcohol (PCI- 25:24:1). This was followed by 2 volumes of ethanol precipitation with $1/10^{th}$ volume of sodium acetate (pH 5.2) and 2 µl glycogen (10 mg/ml) for overnight at -80°C. qRT-PCR was performed as mentioned below and % input calculation was employed to assess the relative enrichment using Ct values.

ΔCt [normalized ChIP] = (Ct [ChIP] - (Ct [Input] - Log2 (Input dilution factor)
Input dilution factor = (Fraction of the input chromatin saved)$^{-1}$ X dilution factor before qPCR.
Input % = $100/2^{\Delta Ct\ [normalized\ ChIP]}$

RNA preparation and c-DNA synthesis

A primary overnight culture was sub-cultured to OD_{600} 0.2. The total RNA was isolated from 5 ml culture of secondary culture at OD_{600} 0.8-1.0 employing hot acidic-phenol method (pH 5.2) as described by Collart and Oliviero[84]. The cells were collected by centrifugation, washed with ice-cold water, and flash-frozen in liquid nitrogen. For time course experiments, the pellets were stored at -80°C until further processing. The pellet was dissolved in 400 µl of TES (10 mM Tris-Cl pH 7.5, 10 mM EDTA, 0.5% SDS) and transferred to a new microfuge tube containing pre-warmed acidic phenol (65°C). The samples were then incubated at 65°C for one hour with intermittent vortexing every 10 minutes. The tubes were then placed on ice for 5 minutes and centrifuged at maximum rpm for 5 minutes (4°C). The top aqueous layer was collected, and an equal volume of acidic phenol was added, vortexed, and centrifuged at maximum rpm for 5 minutes (4°C). The above step was repeated again with an equal amount of chloroform and aqueous layer was recovered. To the above, 2 volumes of 100% ice-cold ethanol and 40 µl of 3M sodium acetate (pH 5.2) was added and precipitated overnight at -80°C. The RNA was then pelleted down by centrifuging at maximum rpm for 5 minutes at 4°C and washed once with 70% ice-cold ethanol. The pellet was air dried at room temperature for 10 minutes and resuspended in 50 µl of RNAse free MilliQ. The integrity of the RNA was assessed by both agarose gel electrophoresis and by measuring A260/280 absorbance using

NanoDrop (Thermo Scientific/2000C). The DNA contamination was removed using RNase-free DNase before cDNA synthesis as mentioned below.

For RNA half-life experiment: The cells were grown to mid-log phase at permissive temperature (25°C), centrifuged, and rapidly shifted to non-permissive temperature (39°C) by adding an equal amount of pre-warmed medium (50°C) and the samples were collected at respective intervals.

For *rat1-1 ts* mutant: The cells were sub-cultured and grown upto OD_{600} 0.8-1.0 at permissive temperature (28°C) and shifted to non-permissive temperature (39°C) for 3 hours. An isogenic, similarly treated wild type was used as a control for data normalization.

For DNaseI digestion, 3 µg of RNA was subjected to digestion with 10 units of RNase-free DNaseI (New England Biolabs) enzyme at 37°C for 3.5 hours to achieve RNA samples entirely devoid of DNA contamination. Before cDNA synthesis, a standard PCR (40 cycles) was performed with primers specific for *ACT1* to assess the DNA contamination and only DNA-free samples were used. RNA alone was also used as a control in qRT-PCR to confirm the purity of the samples. The reverse transcription was performed using Verso cDNA Synthesis Kit (Thermo Scientific) as per the manufacturer's instructions at 55°C for 60 minutes followed by inactivation at 95°C for 2 minutes.

TERRA level analysis by qRT-PCR

TERRA (telomeric repeat-containing RNA) reverse transcription was done using 10 µM of CA oligonucleotide and 2 µM ACT1 oligonucleotide in a final volume of 20 µl reaction. For qPCR, the cDNA was diluted with an equal volume of nuclease-free MilliQ. A volume of 1 µl cDNA was quantified in a final volume of 10 µl reaction by real-time PCR with the Power UP SYBR Green Master mix (Applied Biosystems) using Quanstudio3 Real-Time PCR system (Thermo Scientific). The final concentration of each primer set differed from 0.2–0.6 µM, as described by Iglesias *et al.*, 2011. The reaction was incubated for 10 minutes at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 59°C. TERRA levels were normalized to respective actin values and compared to the isogenic wild type. The primers Y′6/Y′4/Y′3/10R14R detected TERRA stemming from more than one chromosomal end (6Y′-

8 L/8 R/12 L-YP1a /12 R-YP2a /13 L/15 R) (4Y′-9 L/10 R/12 R-YP2a /15 R) (Y′3-12 L-YP1a /12 R-YP2 a /15 R). Whereas for X-only containing telomeres, individual primers were designed for − 4 L, 7 L, 10 R, 13 R, 15 L, 10R14R. The corresponding sequence information is detailed in the list of primers used in this study.

The ΔΔCt method was used to calculate the relative fold change:

ΔCt = Ct (Gene of interest) – Ct (Housekeeping gene)

ΔΔCt = ΔCt (Mutant) – ΔCt (Wild type)

Relative Fold Change = $2^{-(\Delta\Delta Ct)}$

SDS-PAGE and western blotting

The cells corresponding to 2 units of OD_{600} were subjected to protein extraction by TCA method. To the cell pellet, 200 µl of 20% TCA was added and lysed by vortexing at maximum speed for 5 minutes. The lysate was then collected into a fresh tube and glass beads were washed twice with 150 µl of 5% TCA. The washed elutes were then added to the previous lysate and spun at maximum rpm for 5 minutes. To the pellet, 200 µl of Laemmli buffer (200 mM Tris-HCl pH 6.8, 8% SDS, 40% Glycerol, 6 mM bromophenol blue, 4% β-mercaptoethanol) was added and pH was adjusted using un-pHed 2 M Tris. The dissolved protein pellet was then denatured by boiling at 99°C for 5 minutes. SDS-PAGE was performed followed by a semi-dry method of transfer (Power Blotter System/Invitrogen) to PVDF membrane for western blotting. The blots were then probed with primary antibody specific for the epitope tag of interest and its respective secondary antibody conjugated with HRP. The signal was detected using chemiluminescence (ECL reagent/BioRad) and imaged in ChemiDoc Imaging system (BioRad).

Northern hybridization by slot-blotting

For RNA slot blots, 10 µg of denatured RNA was directly loaded onto Hybond N+ membranes (Amersham) using slot blot manifold Hoefer PR648 (Amersham). The RNA was denatured by (2.2 M formaldehyde, 50% formamide, 0.5 X MOPS buffer) heating at 55°C for 15 minutes. The generation of probes and whole hybridization procedure were carried out according to manufacturer's protocol using DIG-High prime DNA labelling and detection starter kit I (Roche).

Polysome profiling

Yeast cells were grown to OD_{600} 1.0 at 28°C in appropriate selection media. The cells were harvested by centrifugation at 4°C in the presence of 100 µg/ml cycloheximide to stabilize the translating ribosomes and flash-frozen in liquid nitrogen. Cell lysis was performed using bead beater (Biospec/Unigenetics) for 3 cycles (1 min ON/OFF) in lysis buffer (20 mM HEPES-KOH pH 7.4, 100 mM potassium acetate, 2 mM magnesium acetate, 0.5 mM DTT, 1 mM PMSF, 100 µg/ml cycloheximide, 1X complete protease inhibitor cocktail). The lysates were then pre-cleared twice by centrifugation at 16,000 *g* for 10 minutes (4°C). The samples were then normalized based on A_{260} absorbance units with lysis buffer, and 100 µg equivalent lysate was loaded onto the 11 ml of pre-chilled sucrose gradient 10-50% (W/V) prepared in lysis buffer. The gradients were then ultracentrifuged in an SW41 Ti rotor (Beckman Coulter) at 39,000 rpm for 2 hours at 4°C. Gradient analysis was performed using Teledyne Isco UA/VIS detector and continuously monitored for A_{254} absorbance values.

RNA sequencing

RNA sequencing was done for two biological replicates. A single colony of yeast was inoculated in 10 ml of YPD and grown overnight at 30°C. The cells were then sub-cultured to OD_{600} 0.8-1.0 and total RNA was isolated from 50 ml culture employing RiboPure™ RNA Purification Kit, yeast (Thermo Scientific). The RNA integrity was assessed by running an aliquot of the samples on an Agilent RNA Bioanalyzer chip. The library preparation and RNA-sequencing were done by Genotypic Technology Pvt Ltd (Bangalore). The differential expression analysis was done in comparison to the wild type via the empirical Bayes model (EBSeq-R). A target FDR of 0.01 was used, and the list of genes obtained was further refined using a minimum fold change cut-off of 2. The percentage of aligned reads was >85%, and the Spearman correlation coefficient was >0.99 between the replicates.

Plasmid isolation from yeast

Transformants carrying the plasmids were inoculated in 5 ml of appropriate selection medium and incubated overnight at 30°C in a shaking incubator. The cells were then harvested by centrifugation and resuspended in 200 µl of breaking buffer (2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0). The suspension was then

transferred to 2.0 ml microfuge tubes containing 0.3 g glass beads (~200 µl volume), and 200 µl of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and vortexed at maximum speed for 3 min. The tubes were then centrifuged at maximum speed for 5 min. The aqueous layer was recovered, and 5-10 µl was transformed directly into ultracompetent *E. coli* cells. Alternatively, for long-term storage, the entire top aqueous layer was transferred into a new microfuge tube and an equal volume of 95% ice-cold ethanol was added to it. The contents were mixed gently by inversion and incubated at -20°C for 30 minutes. The DNA was precipitated by centrifuging at maximum speed for 10 minutes at room temperature. The pellet was then washed with 70% ethanol and air-dried at room temperature to remove the residual ethanol. To the pellet, 20 µl of TE-RNase was added and 10 µl was transformed into 100 µl of ultra-competent *E. coli* cells.

Site-directed mutagenesis

Rtt103 (R108N)*13 Myc were generated through site-directed mutagenesis using the oligonucleotides mentioned in Table 2.3. Site-directed mutagenesis was performed via PCR using KOD-Plus-Neo (Toyobo/Puregene) in 50 µl reaction mixtures containing ~50 ng of plasmid DNA, 0.2 mM dNTPs, 1.5 mM $MgSO_4$, 2 µM of each primer (forward and reverse), 10X KOD-Plus-Neo buffer to a final concentration of 1X, and 1 µl of KOD-Plus-Neo (1 U/50µl). A step-down cycle was employed according to the manufacturer's protocol with an extension time of 1 kb/ min. Then, 20 µl of the PCR product was digested with 1 µl of DpnI (NEB 10 U/µl) overnight at 37°C to degrade the methylated template plasmid. Subsequently, 10 µl of DpnI digested material was transformed into *E. coli* DH5-α ultra-competent cells. The plasmid DNA was then sequenced to confirm the mutagenesis and verified for secondary mutations.

Plasmid transformation into yeast

Yeast transformation was performed using the standard lithium acetate (LiAc) protocol as described by Gietz and Woods[85]. A single colony of yeast was inoculated in 10 ml of YPD or SC and grown overnight at 28°C. The cells were harvested by centrifugation at 4000 rpm for 5 minutes and washed once with sterile double-distilled water. The washed cells were harvested again and resuspended in 1 ml of 100 mM LiAc. Then, 150 µl of the above

suspension was harvested and 350 µl of the transformation mixture (PEG 3500 50%- 240 µl, LiAc 1.0 M-36 µl, SS DNA (2 mg/ml)- 50 µl, plasmid DNA (0.1-1µg) + H_2O-24 µl) was added. The reaction mixture was then vortexed to resuspend the cells and incubated in a thermomixer/water bath at 42°C for 40 minutes. After incubation, the cells were harvested by centrifugation at 6000 rpm for 5 minutes and resuspended in 100 µl of sterile double distilled water. The resuspended mixture was plated on the appropriate selection medium and incubated at 30°C for 2-3 days.

High-throughput transformation of yeast

For high-throughput transformation, the above protocol was scaled up 10X in both cell titre and reaction volume to achieve the desired number of transformants. A single colony of yeast was inoculated in 10 ml of YPD or SC and grown overnight at 28°C. The primary overnight culture was sub-cultured in 100 ml to OD_{600} 0.2. Once the cells reached the mid-log phase (OD_{600} 0.8-1.0), 50 ml of the cells were harvested by centrifugation at 4000 rpm for 5 minutes. The cells were washed twice with 25 ml of sterile double distilled water and resuspended in 3 ml of 0.1 M LiAc. The above cell suspension was aliquoted into 15 ml tubes and incubated at 30°C for 30 minutes. After incubation, the cells were harvested and a transformation mixture (PEG 3500 50%- 2.4ml, LiAc 1.0 M-360µl, SS DNA (2 mg/ml)-500 µl, library DNA (3 µg) + H_2O-240 µl) was added in the same order as mentioned. The mixture was vortexed vigorously until the cell pellet was completely resuspended and incubated at 30°C for 30 minutes. The mixture was then subjected to heat shock at 42°C for 60 minutes. The tubes were either vortexed or inverted several times every 15 minutes to equilibrate the temperature and ensure proper mixing. The cells were pelleted by centrifugation at 4000 rpm for 5 minutes and resuspended in 1 ml of sterile double distilled water. As the cell titer was high, the suspension was spread over five appropriate selection plates (200µl each) to avoid persister colonies and false positives. The plates were then incubated at 30°C for 2-3 days.

For the yeast two-hybrid screen, 3 µg of yeast genomic library was used, which contains 1 µg each of all the three pGAD-C1, pGAD-C2, and pGAD-C3 open reading frame libraries mixed together. The strain used was PJ69-4A, transformed with $pGBKT_7$-RTT103, and confirmed for lack of transactivation. For the cold-sensitivity suppressor screen, 3 µg of genomic library in YEP13 was used. The library was constructed using partially digested

Sau3A fragments of the yeast genome. Both the libraries were received as gifts from Prof. David Shore, University of Geneva, Switzerland.

For PCR-mediated homologous recombination to generate either knock-outs or genomic tags, 5-10 µg of PCR product was used for transformation. For assessing G418 or hygromycin resistance, the cells were resuspended in rich media after heat shock for at least 6 hours at 30°C to allow the expression of the selectable marker.

Rapid isolation of yeast chromosomal DNA

A single colony of yeast was inoculated in 10 ml of YPD and grown overnight at 30°C to stationary phase. The cells were then harvested by centrifugation at 4000 rpm for 5 min. The supernatant was discarded and cells were resuspended in 200 µl breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0). The cell suspension was transferred to a 2.0 mL microfuge tube containing glass beads approximately up to ~200 µl mark and 200 µl of Phenol: Chloroform: Isoamyl alcohol (P:C:I-25:24:1) was added to it. The tubes were then sealed with parafilm and vortexed at high speed for 2 min or beat-beaten in a homogenizer for 1 min at maximum speed. Then, 200 µl of TE pH 8.0 was added to the lysed cells and vortexed briefly for 30 s at maximum speed. The tube was centrifuged at 13200 rpm for 5 min and the top aqueous layer was transferred to a clean microfuge tube containing 1 ml of 100% ethanol. The contents were mixed by gently inverting the tube and the DNA was precipitated by centrifuging at 13200 rpm for 5 min. The pellet was then washed with 70% ethanol to remove the residual salts and then it was air-dried. To the pellet, 100 µl of TE-RNase was added and incubated at 37°C for 30 minutes. The quality of the isolated DNA was assessed by agarose gel electrophoresis and appropriate dilutions were used for downstream applications.

P-body imaging

P-bodies are dynamic and transient structures that can change rapidly in response to a wide variety of stresses. Even centrifugation or different handling times can result in variations in P-body size and number. Hence, logarithmically growing cells (OD_{600} 8.0-1.0) were immediately fixed directly in the medium with 1/10 volume of 37% formaldehyde (3.7% working) at 30°C for 20 min (without shaking). The cells were then subjected to a gentle wash

(twice) and placed on 0.1% concanavalin-coated cover slides and mounted using Slow Fade Anti-fade mounting medium. The images were acquired on Leica TCS SP8 using HC PL APO CS2 63X/1.40 oil objective. The size and number of P-bodies per cell were quantified as described by Nissan and Parker[86].

Polymerase chain reaction (PCR)

For the amplification of DNA fragments, either yeast genomic DNA or plasmid DNA (50-100 ng) was used as a template in 25 µl reactions (10X buffer-2.5 µl, 10 mM dNTPs-0.5 µl, 10 mM forward primer-0.5 µl, 10 mM reverse primer-0.5 µl, template-1 µl, polymerase-0.125 µl, Milli Q-19.875 µl). The oligos used in the PCR reactions were procured from Eurofins and are listed in Table 2.3. For colony PCR, standard Taq polymerase was used, and for cloning, proofreading PCR was performed using Vent polymerase (NEB). The PCR products were assessed using 0.8% agarose gel electrophoresis, and then the bands were excised from the gel and purified using the QIAGEN Gel Extraction Kit according to the manufacturer's protocol for downstream applications.

Colony-PCR

For rapid screening of genomic disruption/tagging or identification of library DNA of interest, colony-PCR was employed. A small number of cells were resuspended in 40 µl of 20 mM NaOH and lysed by boiling at 98°C for 15 minutes followed by chilling on ice immediately. The tubes were then spun at maximum speed for 1 min, and 1 µl of the supernatant was used as the template for a 25 µl PCR reaction.

Restriction digestion

A variety of restriction endonucleases were used in this work for the digestion of PCR products, preparing the defined fragments for sub-cloning, or confirming the correct orientation of clones. All the enzymes used were high-fidelity restriction endonucleases from NEB. Typically, a 10 µl reaction (10X r-Smart Buffer-1 µl, DNA-1-2.5 µg, Enzyme -0.2 µl (4 Units), Milli Q- up to 10 µl) was carried out at 37°C for a minimum of 60 minutes.

DNA ligation

In order to clone desired DNA fragments into vectors of interest, the quantity and quality of the restriction-digested fragments were assessed by both agarose gel electrophoresis and Nanodrop 2000C (Thermo Scientific). A three-fold molar excess of insert compared to the vector was setup in a 10 µl reaction (NEB T_4 DNA ligase Buffer- 1 µl, Vector:Insert-3:1 molar ratio, T_4 DNA ligase-0.5 µl, Milli Q-up to 10 µl) and incubated overnight at 16°C or 4 hours at 24°C. The entire 10 µl ligation mixture was transformed into 100 µl of DH5-α ultra-competent *E. coli* cells.

Concentrating nucleic acids

DNA or RNA was precipitated from an aqueous solution by adding $1/10^{th}$ volume of 3 M sodium acetate (monovalent cation) and 2 volumes of ethanol followed by incubation at -20°C for 2 hours. The nucleic acids were then pelleted by centrifugation at maximum speed for 20 minutes at 4°C. The pellet was washed with 70% ice-cold ethanol to remove the residual salts. The pellet was air dried at room temperature and resuspended in an appropriate amount of TE Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0).

Bacterial growth and media

Conventional media, including LB (0.5% yeast extract, 1% tryptone, 1% NaCl) and SOB (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl and 10 mM $MgCl_2$ (added after autoclaving)) were used for culturing the bacterial strains at 37°C.

Plasmid isolation from bacteria

Plasmid isolation from bacteria was done using alkaline lysis method as described by Sambrook and Russell[87]. A single bacterial colony was inoculated in 5 ml of LB broth containing appropriate antibiotic selection and incubated overnight at 37°C with shaking at 220 rpm. A total of 3 ml of the culture was harvested by spinning at 13200 rpm for 1 min. The pellet was then resuspended thoroughly in 200 µl of solution I (25mM Tris-Cl pH 8.0, 10 mM EDTA pH 8.0) followed by 200 µl of solution II (0.2% NaOH, 0.2% SDS) and mixed gently by

inverting the tube. Then, 200 µl of solution III (3 M potassium acetate, 11.5% glacial acetic acid) was added and the contents were mixed gently by inversion and placed on ice for 5 min. The tubes were then centrifuged at maximum rpm for 10 min at 4°C. The clear supernatant was then recovered and 420 µl of isopropanol was added, mixed by inversion, and incubated at room temperature for 5 min. The DNA was then precipitated by centrifugation at maximum speed for 10 minutes. The pellet was then washed with 70% ethanol to remove the residual salts and then it was air-dried. The pellet was resuspended in 50 µl of TE RNase (10mM Tris-Cl pH 7.4, 1mM EDTA pH 8.0) and incubated at 37°C for 30 minutes. The concentration and quality of the plasmid was assessed by agarose gel electrophoresis.

Preparation of ultra-competent bacterial cells

The ultra-competent DH5-α *E. coli* cells were prepared using the Inoue method as described by Sambrook and Russell[88]. A single colony of bacteria was grown overnight at 37°C and sub-cultured into 25 ml of SOB in a 250 ml conical flask for 6-8 hours. Then, 10 ml of the above starter culture was sub-cultured to OD_{600} 0.55 at 16°C with moderate shaking. The cells were then cooled down by placing them on ice for 10 minutes and harvested by centrifugation at 2500 *g* for 10 minutes at 4°C. The cells were then briefly equilibrated in 80 ml of ice-cold Inoue transformation buffer (50 mM $MnCl_2.4H_2O$, 15 mM $CaCl_2.2H_2O$, 250 mM KCl, 500 mM PIPES pH 6.7) and harvested again by centrifugation at 2500 *g* for 10 minutes at 4°C. The cells were then finally resuspended in 20 ml of ice-cold transformation buffer containing 1.5 ml DMSO and incubated on ice for 10 minutes. Then, 100 µl of the above suspension was aliquoted into 1.5 mL microfuge tubes and snap-frozen in liquid nitrogen. The tubes were then stored at -80°C until further use.

Bacterial transformation

A single aliquot of 100 µl ultra-competent DH5-α cells prepared by the above method was used for transformation. A total of 2 µl of plasmid DNA was added to the cells stored at -80°C, which were then allowed to thaw gradually on ice for 20 minutes. The thawed cells were then given heat shock for 90 s at 42°C and chilled on ice again for 5 min. To the above cells, 900 µl of LB medium was added and incubated at 37°C for 40 min in a thermomixer with shaking at 600 rpm. Then, 100 µl of the above cell suspension was spread on an appropriate antibiotic plate and incubated at 37°C overnight. For transforming the ligated DNA mixture,

900 µl of SOB was used instead of LB medium to improve the efficiency. The entire cell suspension was harvested by centrifugation at 13200 rpm for 1 minute. Subsequently, 900 µl of the supernatant was removed and the cells were resuspended in the remaining medium and spread on an LB plate containing appropriate antibiotics.

2.2 List of strains used in this study

The *Saccharomyces cerevisiae* strains used in this study are derivatives of W303 or BY4741 and are listed below. All the W303 strains carry a wild-type RAD5 gene.

Strain No.	Genotype	Source
KRY 105	W303 *adh4*::*ADE2* Tel VII L MATa	Lab collection
KRY 193	W303 *adh4*::*URA3* Tel VII L MATa	Lab collection
KRY 230	KRY 105 except *rtt103Δ*::KanMx MATα	Lab collection
KRY 285	KRY 193 except *rtt103Δ*::KanMx MATα	Lab collection
KRY 172	KRY 193 except *yku70Δ*::KanMx MATα	Lab collection
KRY 632	BY4739 *rai1Δ*::KanMx *his3Δ1 leu2Δ0 ura3Δ0* MATα	Arlen Johnson
KRY 634	Dat1-1 *rat1-1^{ts} ura3-52 leu2Δ1 his3Δ200 trp1Δ63* MATα	Arlen Johnson
KRY 2178	yRP 693 *ura3–52 leu2 rpb1-1^{ts}*	Carolyn Decker
KRY 2179	KRY 2178 except *rtt103Δ*::KanMx	This Study
KRY 18	*sir2Δ*::KanMx MATα	Lab collection
KRY 931	*ura3, leu2, ade2Δ*, tel-XI coreX::*URA3* MAT-α	E. Fabre
KRY 2275	KRY 931 except *rtt103Δ*::KanMx	This Study
KRY 2182	KRY 2181 except *rtt103Δ*::KanMx	This Study
KRY 2276	BY4741 *rpa49Δ*::KanMx	Euroscarf

KRY 2277	BY4741 *nsr1Δ*::KanMx	Euroscarf
KRY 1708	*rdn1Δ*::2XrDNA pRDN-hyg::*URA3* MAT-a	David Shore
KRY 1770	*bar1*-Δ *lys2*::pGAL-ISCEI Tet"O" p::URA3::ISceI MAT-a	This Study
KRY 1772	KRY1770 except *rtt103Δ*::KanMx	This Study
KRY 2278	KRY 105 except *rai1Δ*::KanMx MAT-α	This Study
KRY 2279	KRY 105 except *rtt103Δ*::KanMx *rai1Δ*::KanMx MAT-α	This Study
KRY 280	pJ694-A *trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*	Lab collection
KRY 2180	BY4741 RTT103-TAP::HIS3Mx6	Horizon Discovery
KRY 2181	BY4741 RAT1-TAP::HIS3Mx6	Horizon Discovery

Table 1: List of strains used in this study.

2.3 List of plasmids used in this study

Plasmid No.	Description	Source
CKM 67	pFA6a-KanMX6	Lab collection
CKM 1	YCplac22 TRP1 (CEN)	Lab collection
CKM 2	YCplac111 LEU2 (CEN)	Lab collection
CKM 5	YEplac112 TRP1 (2μ)	Lab collection
CKM 6	YEplac181 LEU2 (2μ)	Lab collection
CKM 261	CKM 1 with full length RTT103	Lab collection
CKM 233	CKM 6 with full length RTT103	Lab collection
CKM 285	CKM 5 with full length RTT103	Lab collection
CKM 273	pBEVY-T	Lab collection
CKM 287	pBEVY-T + RTT103*13xMyc	This study
CKM 767	pBEVY-T + RTT103 (R108N) *13Myc	This Study
CKM 792	P_{GAL7}-RDN-35S^{WT}	Norbert Polacek/UB
CKM 793	P_{GAL7}-RDN-18S^{WT}	Pankaj Alone/NISER
CKM 794	Edc3-mCherry	Purusharth Rajyaguru/IISc

Table 2: List of plasmids used in this study.

2.4 List of primers used in this study

Primer Name.	Sequence 5′-3′	Purpose
ACT1 F ACT1 R	GTAACATCGTTATGTCCGGTGGTAC CCAAGATAGAACCACCAATCCAGAC	RT-PCR
YIR042-C F YIR042-C R	ATTTGGGCAAGAAGTTGGTG TTCCGAACCATGTCTTTCTCT	RT-PCR
YFR057-W F YFR057-W R	CTAGTGTCTATAGTAAGTGCTCGG CTCTAACATAACTTTGATCCTTACTCG	RT-PCR
Y'6- F Y'6- R	GGCTTGGAGGAGACGTACATG CTCGCTGTCACTCCTTACCCG	RT-PCR
Y'4- F Y'4- R	GGCTTGGAGGAGACGTAAATG CCAACTCTCTCTCATCTACCTTTACTCG	RT-PCR
Y'3- F Y'3- R	GGCTTGGAGGAGACGTACATG CCACACACTCTCTCACATCTACCTC	RT-PCR
4- F 4- R	GGAGTGGA TGGTTGAGTGGGG CTAACACTACCCTATTCTAACCCTGATTTT	RT-PCR

7L- F 7L- R	ACGGTTATGATGGGCGGTGGA CTACCCTAACCCTATTCTAACCCAGATC	RT-PCR
10R- F 10R- R	CGGTTATGGTGGACGGTGGATG CCTAACCCTATTCTAATCCAACCCTGATAA	RT-PCR
13R- F 13R- R	ACGGTTATGGTGCACGATGGG TTACCCTCCATTACGCTACCTCC	RT-PCR RT-PCR
15L- F 15L- R	GGGTAACGAGTGGGGAGGTAA CAACACTACCCTAATCTAACCCTGT	RT-PCR
10R14R- F 10R14R- R	GGATGGTGGTTGGAGTTGTAGAATG ATCCAACCCTGATAAACCTGTCTCTT	RT-PCR
SCR1- F SCR1- R	GGCAGGAGGCGTGAGGAATC CCTAACAGCGGTGAAGGTGGAG	RT-PCR
RPA190- F RPA190- R	GTGATAACTGTGGTATGTTTTCGCC CTGGTGGTAGGATTTCTACCAACG	RT-PCR
RPA135- F RPA135- R	TCAATGACAAGTTCCAAGTTCGTTC TGGTCAAAATAGAACCACACTCGC	RT-PCR

RPA49- F RPA49- R	ATCCAAGTCAAGCAAGAATCTAAGGG ATTGAGCCCGAGTTGGTAAATCC	RT-PCR
RPA34- F RPA34- R	AAACGAGTTCAGCATACCAGATGG ACGAGGACTCAATATCTGTATCGTCC	RT-PCR
RPC31- F RPC31- R	GATGAAAACATCGGACTATCCATGC CATCACCGTAATCATCATCGTCACC	RT-PCR
RPC34- F RPC34- R	GCCAGATGATGTCGAAAGGAATAGG GAATATACCAGTGCCTCTTCAGCC	RT-PCR
RPC40- F RPC40- R	GCATTTTAGGTATCGGTGGTGATCAT CGTATCTTAAAACCTCCCTGGAAACC	RT-PCR
RDN18- F RDN18- R	CAAGGCTGAAACTTAAAGGAATTGACG CACCACTATTTAGTAGGTTAAGGTCTCG	RT-PCR
RDN25- F RDN25- R	CAACTTAGAACTGGTACGGACAAGG TCCATTCATGCGCGTCACTAATTAG	RT-PCR
POL5- F POL5- R	GAAGGTGATGAAAGTCTAATTCCCTCG GTAGCAGAACACTCCAAACAAAATGC	RT-PCR

SNR86- F SNR86- R	ATATATGTGCCTTGCTATGAACGCG CACGACTGAAAAACTCAAACGTGC	RT-PCR
RNH202- F RNH202- R	AACGGATAACCATGACAAGAATTGGG GTAGCACTGTTTTATTTCCGTTGGG	RT-PCR
TLC1- F TLC1- R	GTGTCCTTTCTTAAGCATCGGTTAGG GGAGAAGTAGCTGTGAATACAACACC	RT-PCR
DBP2- F DBP2- R	ACACGATATTCCAAAGCCAATCACC TGGTTGAGCGTTGATATGAACAATACC	RT-PCR
TPA1- F TPA1- R	GCTGGTAAATTGTCTGGTTCTAAGACC GGAACTAGTTTAGCAGATGGATCGG	RT-PCR
RNT1- F RNT1- R	GCCTCCAAAATTACCAGAGATTCAAG CCTTAATGTTGATAACTGACCCTCGC	RT-PCR
NRD1- F NRD1- R	CATCGATTCAATAGGTAGAGCTTACTTGG GTTCAAGTAACTCTTTTGAAACAAGCCG	RT-PCR
ATG39- F ATG39- R	GAACGGTATTGCCAAGTATGGAGG TAATGACTGTTGTGATTTAAAACCTGCC	RT-PCR

URA3 FP –Tet HR URA3 RP –Tet HR	ATTACCTTTTTTGCGAGGCATATTTATGGTGA AGGATAAGCAGCTGAAGCTTCGTACGC 5'GGATGAGTAGCAGCACGTTCCTTATATGTA GCTTTCGACATATAGGCCACTAGTGGATCTG	For construction of URA3 promoter replacement system
WT Tet-O FP WT Tet-O RP	CGAGCAGAAGGAAGAACGAAGGAAGG GGAGCCCTTGCATGACAATTCTGCTAA	Screening PCR for promoter replacement system
RS Tet-O FP RS Tet-O RP	CTGGATCGGTCCCGGTGTCTTCTATGG GGAGCCCTTGCATGACAATTCTGCTAA	Screening PCR for promoter replacement system
F1-pGBKT7 FL R1 Primer- pGBKT7	CGCATATGCCTTTCTCTTCTGAGCA CGCTGCAGGGTGTCCATGTAGTTTAGGT	For cloning of full length Rtt103 (Yeast two hybrid)
CIDΔ Myc FP CIDΔ Myc RP	AGCGTCGACATGTCCAAGCAGGTAGTCAATG ACATAG ATACTGCAGTACGGGCGACAGTCACATCATG C	For cloning of CIDΔ - Rtt103.
GBD seq primer /pBEVY-T	CGGTAGGTATTGATTGTAATTCTG	For Sequencing
T7 pro seq primer /pGBKT7	GTAATACGACTCACTATAGGGCGA	For Sequencing

Table 3: List of primers used in this study.

CHAPTER 3

The transcription termination complex, Rtt103-Rai1-Rat1, regulates sub-telomeric transcripts in *Saccharomyces cerevisiae*

3.1 Introduction

Telomeres are specialized nucleoprotein complexes that define the ends of eukaryotic linear chromosomes. They play a major role in preserving genome integrity by preventing unscheduled DNA damage repair events at the chromosome ends and also compensate for the inability of DNA polymerase to extend ends of linear chromosomes, the so-called "end-replication" problem[89,90]. Telomeres in most organisms are characterized by the presence of short repetitive sequences that are GT-rich on one strand. The length of these telomeric repeats can vary across species, ranging from approximately 350 ± 75 base pairs (bp) in yeast ($C_{1-3}A/TG_{1-3}$) to several kilobases (kb) in mammals $(TTAGGG)_n$[91]. These repeat sequences are extended by self-templated reverse transcriptase, and the length is maintained within a narrow range[92]. While the exact sequence of the telomere repeats may vary between organisms, their fundamental structure and functions remain conserved across all eukaryotes.

DNA sequences proximal to the telomeric repeats are called telomere-associated sequences (TAS), which consist of both repetitive and coding sequences in most eukaryotes. In yeast, TAS can be broadly classified into two classes, namely X and Y' elements. Y' elements exist in two variants, either long (6.7 kb) or short (5.2 kb), and can be found in 1 to 4 copies per chromosomal end[93]. Y' elements are present in only about half of the telomeres, while X-elements are found in all telomeres. X-elements exhibit greater heterogeneity in both sequence and size compared to Y' elements and are present proximal to the telomeres (Figure 3.1). Additional telomeric repeats may be found at the X-Y junctions in many yeasts[93].

The sub-telomeric regions, which are located near the telomeres, are highly dynamic and undergo frequent recombination events. This dynamic nature leads to significant variations in size, even among closely related strains. Due to these variations, the proteins that bind to the TAS elements differ from telomere to telomere, possibly conferring distinct functions[94,95]. X-

elements are typically transcriptionally repressed and lack nucleosomes, while Y' elements are transcriptionally active and contain nucleosomes. Moreover, Y' elements do not display the typical features of heterochromatin, such as a high occupancy of Sir3 and Rap1 proteins. Additionally, they exhibit low levels of histone H4 lysine 16 acetylation[96].

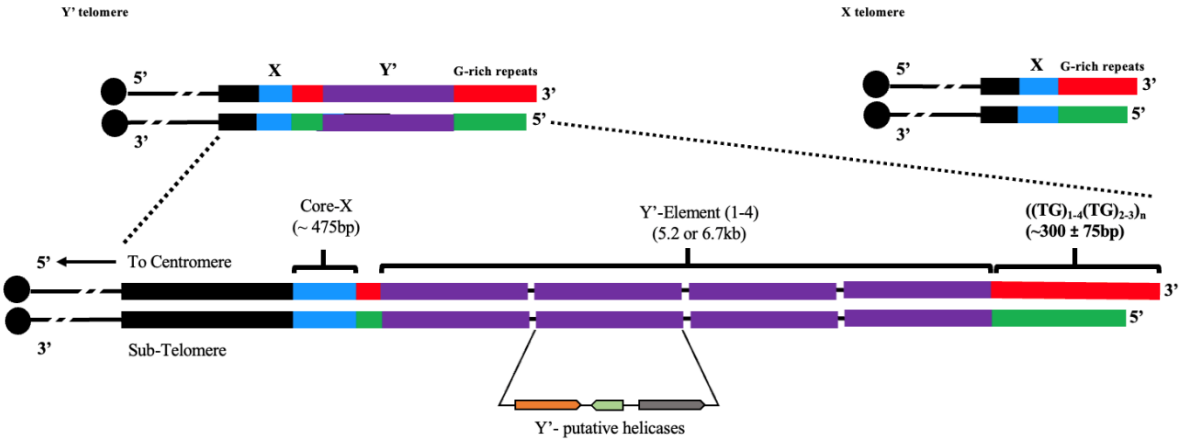

Figure 3.1: Schematic representation of X and Y′ containing yeast telomere: The Y′ elements are either short or long (1-4 copies) arranged in tandem. The TG-rich strand has an extended 3′ single strand overhang when compared to its 5′ complementary strand.

3.2 Telomere Position Effect (TPE)

The genomic regions adjacent to telomeres are usually transcriptionally repressed, which is classically referred to as telomere position effect (TPE)[97,98]. This is due to the establishment of heterochromatin by a subset of telomere resident proteins[91,99]. A diagrammatic representation of telomere region with the majority of proteins known to associate with the telomeres is shown in Figure 3.2. Among them, the major constituents that contribute to TPE in yeast are the double-stranded ($TG_{1\text{-}3}$) binding protein Rap1, DNA end binding Yku complex (Yku70-80), and SIR complex (Sir2-4)[100,101]. Sir4 is recruited by the combined action of Rap1 and Yku70/80, which in turn recruits Sir2, an NAD-dependent histone deacetylase that deacetylates histones H3 and H4[101,102]. Deacetylated histones are bound by Sir3p and Sir4p which further recruits more Sir2 leading to the spreading of this complex towards sub-telomere

up to approximately 3 kb. This epigenetic silencing, once established, remains stable over several cell divisions. Silencing originating from the telomeric tracts exhibits a discontinuous pattern. Although the X-element is effectively silenced in a Sir-complex dependent manner, the Y'-elements are not. Further, genome-wide transcript analysis reveals that only a limited number of genes located more than 3 kb away from the telomere tracts are silenced in a Sir-dependent manner, while the majority are not bound by Sir proteins[103]. However, the transcript levels from these regions are generally low.

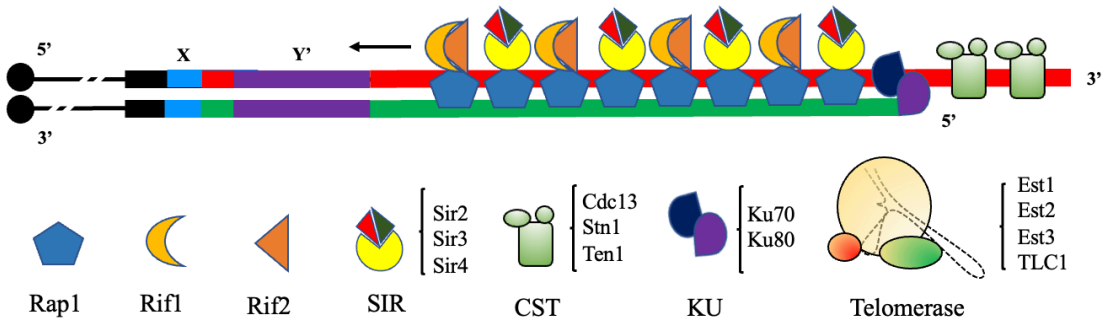

Figure 3.2. **Schematic representation of the telomere resident proteins:** Rap1, a sequence specific DNA binding protein, binds to the telomeric (TG_{1-3}) tracts and recruits both Sir complex (Sir2,3,4) and Rif (Rif1 and Rif2) to the telomeres. The Yku70/80 complex binds to telomeric dsDNA. CST complex associates with the terminal ssDNA overhang and protects the ends of the chromosome. The telomerase complex consisting of the RNA template (TLC1), telomerase reverse transcriptase, Est2, and associated proteins, Est1 and 3 are recruited to the telomeres during telomere replication.

With the advent of new sequencing technologies, now it is clear that despite the epigenetic silencing, several RNA species arise from the telomeric and sub-telomeric region. These include TERRA (telomeric repeat-containing RNA), ARRET, ARIA, sub-TERRA, sub-telomeric CUT′s, sub-telomeric XUT′s, and several other RNA species of telomeric origin[104,105]. Further genome-wide analysis of RNA Pol II enrichment revealed its association with various epigenetically silenced regions, including telomeres, centromeres, rDNA 'E-pro' region, HML, and HMR loci, suggesting that epigenetic mechanisms may not be able to completely prevent transcription from the "silenced" loci[106,107]. Further, it was demonstrated

that the transcripts arising from these loci are co-transcriptionally degraded in conjunction with TRAMP and exosome complex[107]. These studies further illustrate that there might be an additional level of co-transcriptional control that exists and regulates the levels of RNA arising from such epigenetically silenced regions.

TERRA is a class of RNA arising from the telomeres, and is conserved from yeast to humans[108–110]. In *S. cerevisiae,* TERRA is transcribed by RNA Pol II and consists of a heterogeneous population of RNA comprising XY′ or X sequences and telomeric G-strand transcripts. Levels of TERRA are tightly regulated by both epigenetic and post-transcriptional mechanisms[104,111,112]. In X-only containing telomeres, the repression of TERRA transcription is mediated via both Sir2/3/4 complexes and Rap1-Rif1/2 complex described above. In Y′-containing telomeres, Rap1-Rif1/2 complex primarily represses transcription of TERRA[111]. Irrespective of its origin, the turnover of TERRA is tightly regulated by Rat1, a nuclear 5′-3′ exonuclease as Rat1 mutants have increased abundance of TERRA transcripts[111–113]. While our understanding of the exact molecular mechanism of TERRA regulation still remains elusive, it is clearly regulated by both transcriptional and post-transcriptional mechanisms. Besides, TERRA levels are also cell-cycle-regulated and optimal levels appears to be crucial for cellular fitness as either downregulation or overexpression causes telomere dysfunction induced foci[113–119]. According to the current understanding, TERRA plays a significant role in the telomeric architecture as it remains physically associated with the telomeres[113–115].

3.3 Rationale of the study

Previous investigations from our laboratory to identify suppressors for *yku70*Δ temperature sensitive phenotype have yielded high copy expression of *RTT103* as a partial suppressor for both temperature and MMS sensitivity[81]. The yeast Yku complex is an evolutionarily conserved heterodimer and consists of Yku70p and Yku80p subunits[120]. This dimeric complex has a high affinity for terminal regions of double stranded DNA and plays a major role in Non-Homologous End Joining (NHEJ) and maintenance of telomere length. At the telomeres they perform multiple functions: 1. Involved in the recruitment of telomerase RNA and regulates telomere addition[121] 2. Protects the telomeres from recombination events and exonuclease mediated attrition[122] 3. Anchoring of telomeres to the nuclear periphery[123] 4.

Silencing of telomere proximal genes (TPE)[124]. Loss of either subunit of the Yku complex results in complete loss of silencing (TPE). Hence, this work was initiated to assess whether Rtt103 has any role in telomere metabolism or gene silencing.

3.4 Results

rtt103Δ mutants are defective in sub-telomeric silencing

As an initial test of whether Rtt103 contributes to TPE, we assessed the expression of *URA3* placed adjacent to the telomeric tract of chromosome VII L. *rtt103*Δ mutant harbouring *URA3* at the *ADH4* locus, the gene closest to the VII-L telomere, was constructed (Figure 3.3A). In the wild type, this locus is usually silenced by TPE. The loss of silencing was assessed by growing the indicated strains overnight on the selection medium to retain the plasmids and spotted on plates containing 5-fluoroorotic acid (5-FOA). *URA3* encodes for orotidine-5′-phosphate decarboxylase, which is usually involved in pyrimidine biosynthesis. In contrast, if 5-FOA is added to the media, this enzyme converts it into a toxic product, namely 5′-Fluorouridine monophosphate, which restricts the growth of the cell. Hence, the loss of silencing results in the restriction of growth on plates containing 5-FOA. In the wild type, the silencing at the telomeres is stochastic and reversible; hence, cells grew on both SC-URA and SC- FOA plates. However, in *rtt103*Δ, we found a reduction in silencing, as indicated by the reduced growth on 5-FOA plate (Figure 3.3A). *yku70*Δ, which results in the complete loss of silencing, was employed as a positive control and was found to be severely defective for growth on 5-FOA plates. Complementation with either single copy (CEN) or multicopy (2 micron) *RTT103* restored the silencing (Figure 3.3A).

Similarly, we used a color-based assay to assess the loss of silencing by integrating *ADE2* marker at the *ADH4* locus adjacent to telomeric tract on chromosome VII L. *ade2*Δ cells displayed a red color due to the accumulation of P-ribosylamino imidazole (AIR), an intermediate in the adenine biosynthesis pathway. In the wild type, the expression of *ADE2* was metastable, altering between silenced and expressed state, and thereby resulting in a variegated colony color phenotype (Figure 3.3B first plate)[125]. In *rtt103*Δ, 90% of the cells

appeared white with no red sectors due to the loss of silencing (Figure 3.3C). Complementation with single copy (CEN) *RTT103* partially restored the silencing defects.

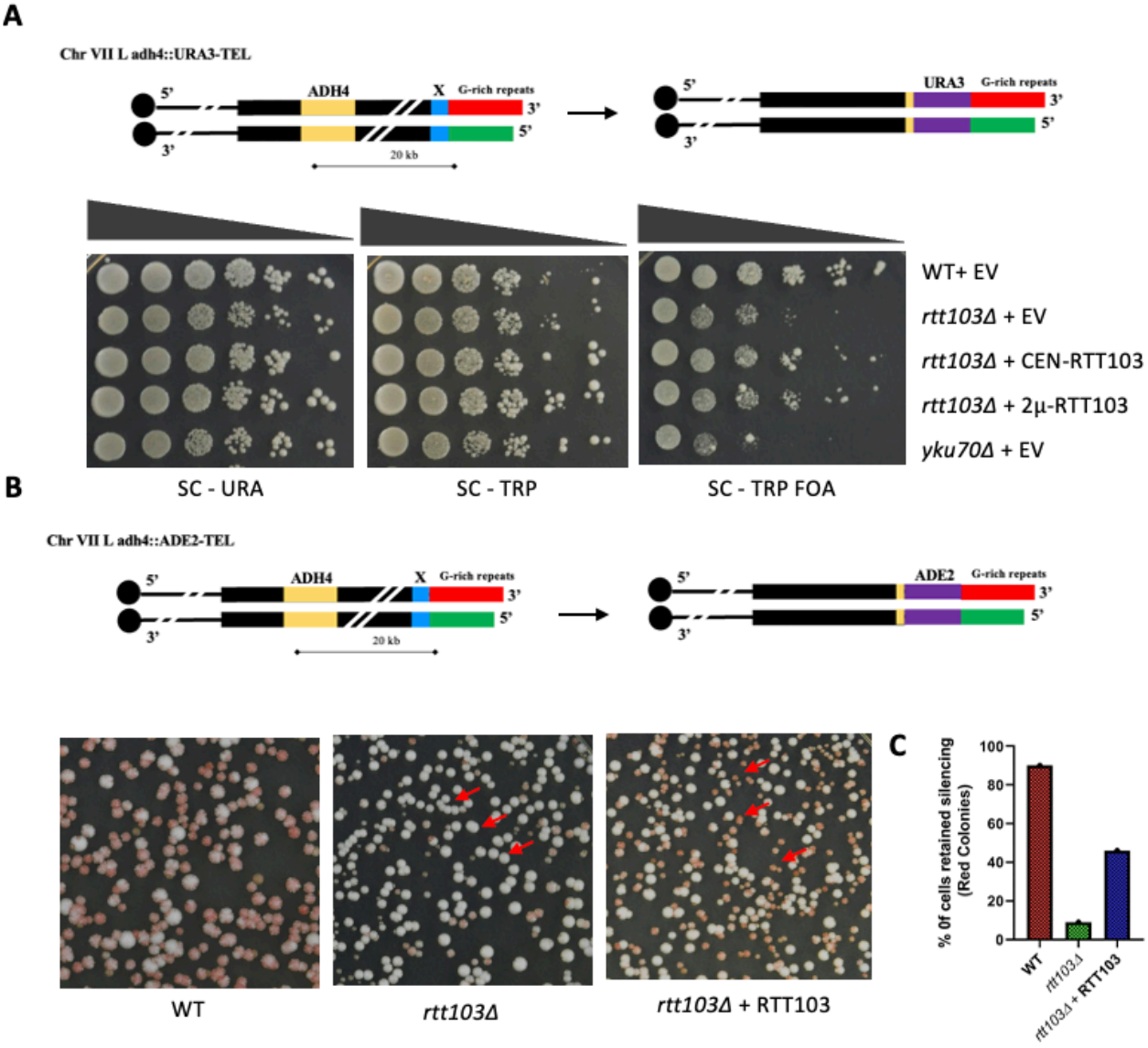

Figure 3.3. ***rtt103Δ* is defective in sub-telomeric silencing at modified telomeres: A.** WT, *rtt103Δ, yku70Δ* transformed with either empty vector (EV) or full length *RTT103* encoded on a single copy CEN vector (CEN) or multicopy 2-micron vector (2μ) were grown overnight and 5 µl of 10-fold serial dilutions were spotted on SC-TRP, SC-TRP, and SC- TRP+FOA plates. Plates were imaged after 72 hours of growth at 30^0C.

B. Wild type, *rtt103Δ*, and *rtt103Δ* carrying full length *RTT103* in CEN vector were grown overnight in synthetic complete media, serially diluted 10-fold and 100 μl was plated on YPD. Plates were imaged after 72 hours of growth at 30°C.

C. Bar graph depicting quantification of percentage of cells that retained silencing (red colonies) (n=500).

Multiple studies have indicated discrepancies in the degree of silencing observed between natural telomeres and modified truncated telomeres. TPE was initially investigated by integrating either *URA3* or *ADE2* adjacent to the telomeric $TG_{1\text{-}3}$ tract via removal of neighboring X and Y' elements as employed in the abovementioned experiments[125]. Although yeast possesses only two types of sub-telomeric transcripts, TPE varies substantially from telomere to telomere in the same cell and between different strain backgrounds[111,126,127]. This is mainly due to the differences in the affinity for proteins that limits or promotes silencing to the sub-telomeric region. Therefore, to verify that the observed silencing defect in *rtt103Δ* is not limited to modified truncated telomeres, we further examined the silencing impairment of a *URA3* marker inserted at the core X region of unmodified natural telomeres on chromosome XI-L[128]. As shown in Figure 3.4A, *rtt103Δ* showed a reduced growth on 5-FOA plates in comparison to wild type, thereby indicating reduced silencing at the native telomeres as well.

We also extended the study to assess the relative transcript levels of two native sub-telomeric genes, namely *YIR042-C* and *YFR057-W* via quantitative reverse transcriptase PCR (qRT-PCR). *YIR042-C* is located at chromosome IX and 3.9 kb away from the telomere end, whereas *YFR057-W* is located at chromosome VI at 645 bp away from the end and have been shown to be silenced in a Sir-protein-dependent manner. qRT-PCR results revealed that in *rtt103Δ*, a 12-fold increase in the relative amounts of *YFR057-W* in comparison to WT was observed. Whereas for *YIR042-C*, only a modest increase in relative levels (statistically not significant) was seen (Figure 3.4B). Complementation with either a single copy (CEN) or multicopy (2μ) *RTT103* restored silencing completely. Collectively, the results obtained from the abovementioned experiments provide compelling evidence that silencing at the sub-telomeres is impaired in the *rtt103Δ* mutant in comparison to the wild type.

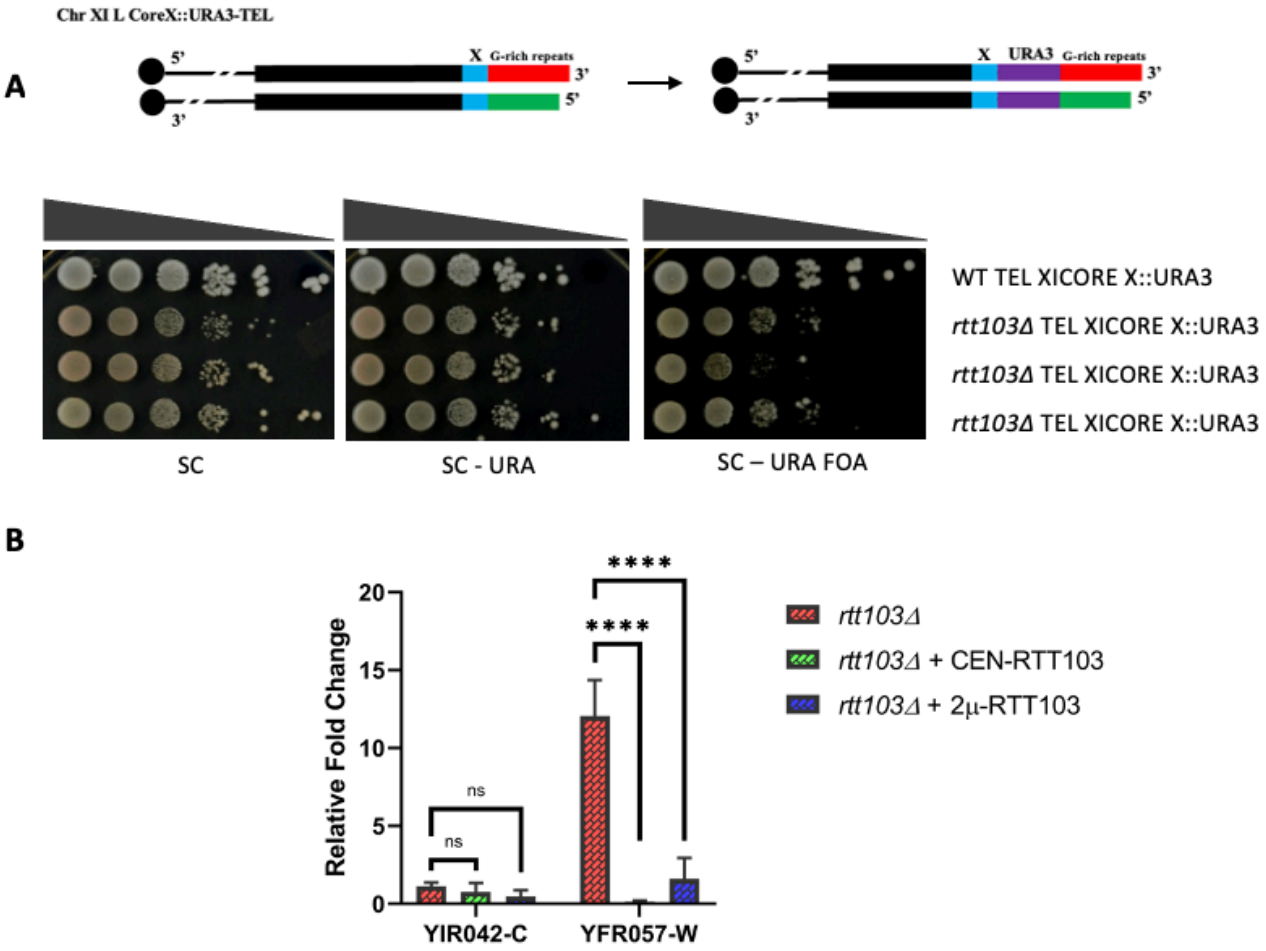

Figure 3.4. ***rtt103Δ* is defective in sub-telomeric silencing at natural telomeres: A.** WT and *rtt103Δ* harbouring TEL XICORE X::URA3 were grown overnight in synthetic complete media, serially diluted 10-fold and 5 µl was spotted on SC, SC-URA, and SC-FOA plates. Plates were imaged after 72 hours of growth at 30°C.

B. RNA was isolated from the indicated strains and converted to cDNA. cDNA was then subjected to qRT-PCR to assess the relative expression of YIR042-C and YFR057-W. All data are depicted as mean + SEM; n=3. P values were obtained from two-way ANOVA.

rtt103Δ, rai1Δ, and rat1-1 are defective in sub-telomeric silencing

Given the proposed coordinated function of Rtt103, Rat1, and Rai1 in transcription termination, we extended the experiments to explore the contribution of this complex to telomere silencing. We assessed the level of silencing defect in *rai1Δ* and *rat1–1* at the two native sub-telomeric genes, namely, *YIR042-C* and *YFR057-W*, via quantitative reverse-transcriptase PCR (qRT-PCR). *RAT1* is an essential gene and the *rat1–1* allele is temperature sensitive. Therefore, the silencing was assessed at 39°C in *rat1–1* and normalized to a wild type isogenic strain grown at 39°C for 3 hours. All three mutants revealed a varying range of silencing defects for *YFR057-W* in comparison to the wild type (Figure 3.5) with *rai1Δ* showing modest increase in transcript abundance. The mutant of Sir2, which is directly involved in epigenetic silencing of the tested sub-telomeric loci, was used as the positive control. Again, *YIR042-C* transcript levels were increased slightly in *rtt103Δ* and *rai1Δ* but showed a more pronounced increase in *rat1–1* and *sir2Δ*. The increased accumulation of telomeric and sub-telomeric transcripts observed upon the loss of all three proteins (Rtt103, Rai1, and Rat1) suggests that these proteins may collectively contribute to telomeric function, similar to their role in transcription termination.

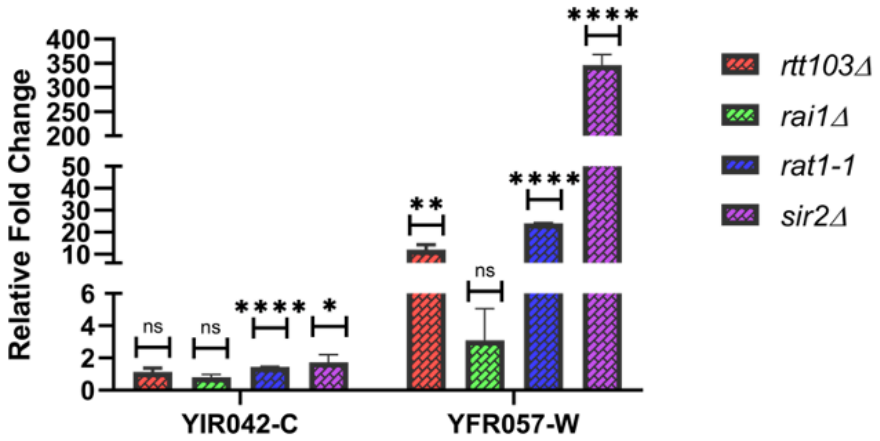

Figure 3.5: ***rtt103Δ*, *rai1Δ*, and *rat1-1* are defective in sub-telomeric silencing:** Relative levels of *YIR042-C* and *YFR057-W* were assessed by qRT-PCR in the indicated strains. Fold

change with respect to the wild type is shown. All data are depicted as mean + SEM; n=3. p values were obtained from Student's t-test.

rtt103Δ alters the stability of sub-telomeric transcripts

Based on several studies, it is now evident that sub-telomeric transcripts are regulated epigenetically, transcriptionally, and post-transcriptionally via RNA surveillance pathways[98,104,112,129]. To delineate whether it is the exacerbated transcription rate (transcriptional regulation) or stability of the sub-telomeric transcripts (post-transcriptionally) that is altered in the *rtt103Δ*, we employed a temperature-sensitive allele of the RNA Pol II subunit, *rpb1–1*. The transcription by RNA Pol II can be selectively turned off by shifting the cultures from permissive (25°C) to non-permissive temperature (39°C), whereas RNA Pol I and Pol III transcription status remained unaltered. We analyzed the stability of transcripts arising from *YFR057-W* and *YIR042-C* along with *ACT1* at regular intervals after inhibiting transcription. Total RNA was isolated from samples at time 0 minutes (arbitrarily set to 1) and every half an hour after the temperature shift to 39°C. Relative abundance of sub-telomeric transcripts and *ACT1* transcripts was obtained by normalization to 7S RNA and plotted as a function of time. 7S RNA is a non-coding RNA arising from the *SCR1* locus, which is transcribed by RNA Pol III, and is unlikely to be affected by defective RNA Pol II function at higher temperatures. In comparison to the wild type *(rpb1–1)*, *rtt103Δ rpb1–1* revealed greater stability of sub-telomeric transcripts, whereas stability of *ACT1* transcript remained the same in both (Figure 3.6A and B). These data suggest that in the *rtt103Δ* mutant, it is the stability of the transcript, not the transcription rate, which is altered and that this is also specific to sub-telomeric transcripts.

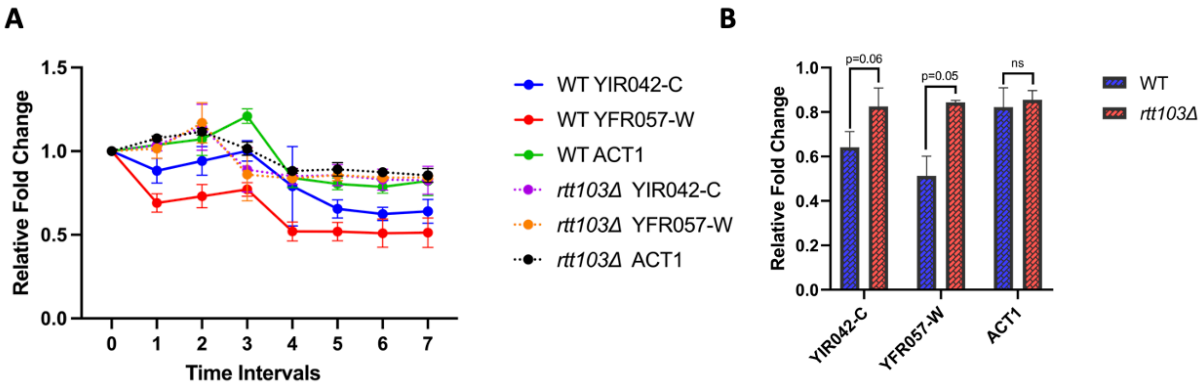

Figure 3.6: ***rtt103Δ* alters the stability of sub-telomeric transcripts: A.** qRT-PCR analysis of the indicated sub-telomeric transcripts in *rpb1-1*(WT) and *rtt103Δ rpb1-1* after shifting to non-permissive temperature. RNA isolated from 0-time point sample was arbitrarily set to 1. *ACT1* was used as the control. The data from two independent experiments are plotted, and the error bars represent the standard deviation at each time point.

B. Relative abundance of *YIR042-C*, *YFR057-W*, and *ACT1* transcripts for the last time point was assessed in the indicated strains. All data are depicted as mean. p values were obtained from Student's t-test (un-Paired).

TERRA accumulates in *rtt103Δ, rai1, and rat1-1*

From our global gene expression analysis in *rtt103Δ* strain via RNA-Seq analysis (described in chapter 4), we found that several of the putative Y′ helicases encoded within Y′-element of the sub-telomeric region were up-regulated by more than two-fold. In *S. cerevisiae* a total of 11 Y′-long elements and eight Y′-short elements exist[130]. Each encompasses more than one open reading frame (ORF), which are annotated as "putative helicases" (Figure 3.1). We first tested the upregulation of Y′ elements in *rtt103Δ* strains via RT-PCR using single primer set, which measures highly conserved Y′ helicases from seven different loci. We indeed found Y′ helicases are upregulated in both *rtt103Δ* and *rai1Δ* (Figure 3.7A and B). Telomeres undergo progressive attrition upon every cell division because of the end replication problem[90]. This is counteracted by the action of telomerase, which employs an RNA moiety to reverse-transcribe and lengthen the telomeric ends[92,131]. In cells lacking telomerase or in cells

undergoing premature senescence, either amplification of Y′ (Type I), TG_{1-3} repeats (Type II) or Y′/TERRA transcripts is utilized to lengthen the telomeres[113,130,132,133].

TERRA is a major class of non-coding RNAs that is produced from the sub-telomeric region and is conserved from yeast to humans[108]. It is thought that the ORFs of Y′ elements encompass promoter-like elements for TERRA[105,134]. The TERRA sequence overlaps with the Y′ helicases and ranges from ∼100–1200 bases in size in *S. cerevisiae*. In yeast, TERRA is an RNA Pol II product and has a 3′ poly-A tail[104,112]. Its expression is tightly regulated via Rap1p- and Rat1p-dependent mechanism and RNA surveillance pathways[104,112,113]. TERRA mostly remains associated at the telomeres suggesting its potential role in telomere replication and architecture; however, the precise function still remains elusive[108,135,136]. Therefore, we assessed the levels of TERRA from multiple chromosomes in *rtt103Δ*, *rai1Δ*, and *rat1–1* as described by Iglesias *et al*. using quantitative real-time PCR protocol[111] (Figure 3.7C). We further confirmed the data by slot blot for TERRA stemming from Y′6-(8L/8R/12L-YP1/12R-YP2/13L/15R) and 15L (Figure 3.7D). We found that TERRA levels were increased in all three mutants and *rai1Δ* had a greater level of accumulation compared to both *rtt103Δ* and *rat1-1*. While it is known that Rat1 plays a key role in maintaining low TERRA levels, we observed that both its partners, Rtt103 and Rai1, also contribute to regulating TERRA levels. These results suggest that as proposed for transcription termination, the Rat1-Rai1-Rtt103 complex could work together in the regulation of TERRA.

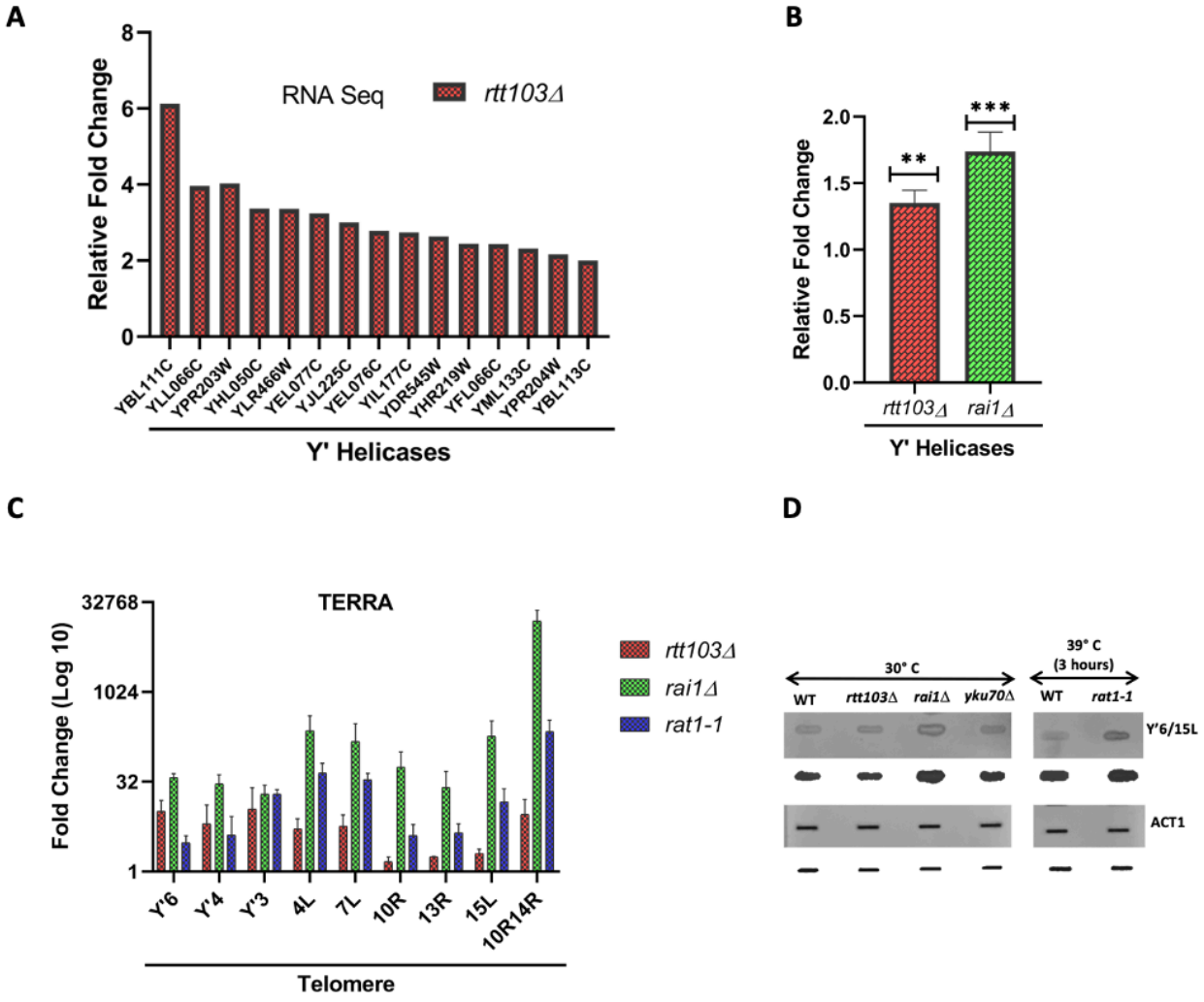

Figure 3.7: **TERRA accumulates in *rtt103Δ*, *rai1Δ*, and *rat1-1*: A.** Global gene expression analysis for various putative Y′ helicases of sub-telomeric origin in *rtt103Δ*.

B. A single primer set that measures highly conserved Y′ helicases from 7 different loci was used to analyse expression by qRT-PCR. Experiments were done for 3 independent colonies and p values were obtained from one-way ANOVA.

C. RNA was isolated from the indicated strains, and RT-PCR with TERRA-specific primers was performed to assess the levels in Y′ and X-only containing telomeres. Three independent experiments were done, and an average of the three with standard error is plotted. The primers Y′6/Y′4/Y′3/10R14R detect TERRA transcripts from more than one chromosomal end.

D. TERRA levels were assessed by slot blot from the indicated strains using probes specific for Y′6 and 15L. Actin was used as the loading control.

Rtt103p recruits Rat1p to telomeres in a transcription-dependent manner

It has been reported that Rat1p associates with the telomeres during late S-phase when the telomere gets replicated, and that this association is dependent on the continued presence of Rif1 and Rif2. The association is abolished upon telomere shortening[113]. Moreover, recent work to identify telomere-associated proteins in *S. cerevisiae* via telomere-mimetic sequence as bait has revealed that both Rat1 and Rai1 are physically associated at telomeres[136]. In transcription termination, Rtt103 has been proposed to be a recruiter for Rai1 and Rat1 by virtue of its interaction with RNA Pol II[47,48]. We, therefore, tested whether Rtt103 also acts as a recruiter of Rat1 to the telomeres. We generated *rtt103Δ* in strains encoding TAP-tagged Rat1. TAP-ChIP was performed in Rtt103-TAP, Rat1-TAP, and *rtt103Δ* Rat1-TAP strains and association with a few telomeric loci was measured. First, we found that Rtt103 is enriched at the telomeres to similar extents as that of Rat1. Second, in the absence of Rtt103, much lower levels of Rat1 could be detected (Figure 3.8A). As the Rat1 protein levels remain unaltered in *rtt103Δ* (Figure 3.8B), it is the recruitment of Rat1 to the telomeres that is impaired significantly.

To further delineate whether this recruitment is transcription-dependent, we performed ChIP for Rat1 in the presence of thiolutin, a well-known inhibitor of yeast RNA polymerases[137]. Upon inhibition of transcription, a substantial reduction in the enrichment of Rat1p was observed at most of the tested telomeric loci (Figure 3.8C). We also performed ChIP with an already known point mutant of *RTT103 (R108N)*, which has reduced interaction with RNA Pol II as determined by anisotropy and NMR measurements[138]. The enrichment of Rat1-TAP at the telomeres was reduced in the strains harboring *RTT103 (R108N)* in comparison with the strain with wild-type *RTT103* (Figure 3.8D) without any reduction in the total amount of Rat1-TAP protein in both strains (Figure 3.8E). This suggests that the recruitment of Rat1p to telomeres is affected and strengthens the idea that at least part of the Rat1 recruitment to telomeres occurs via Rtt103 in a transcription-dependent manner.

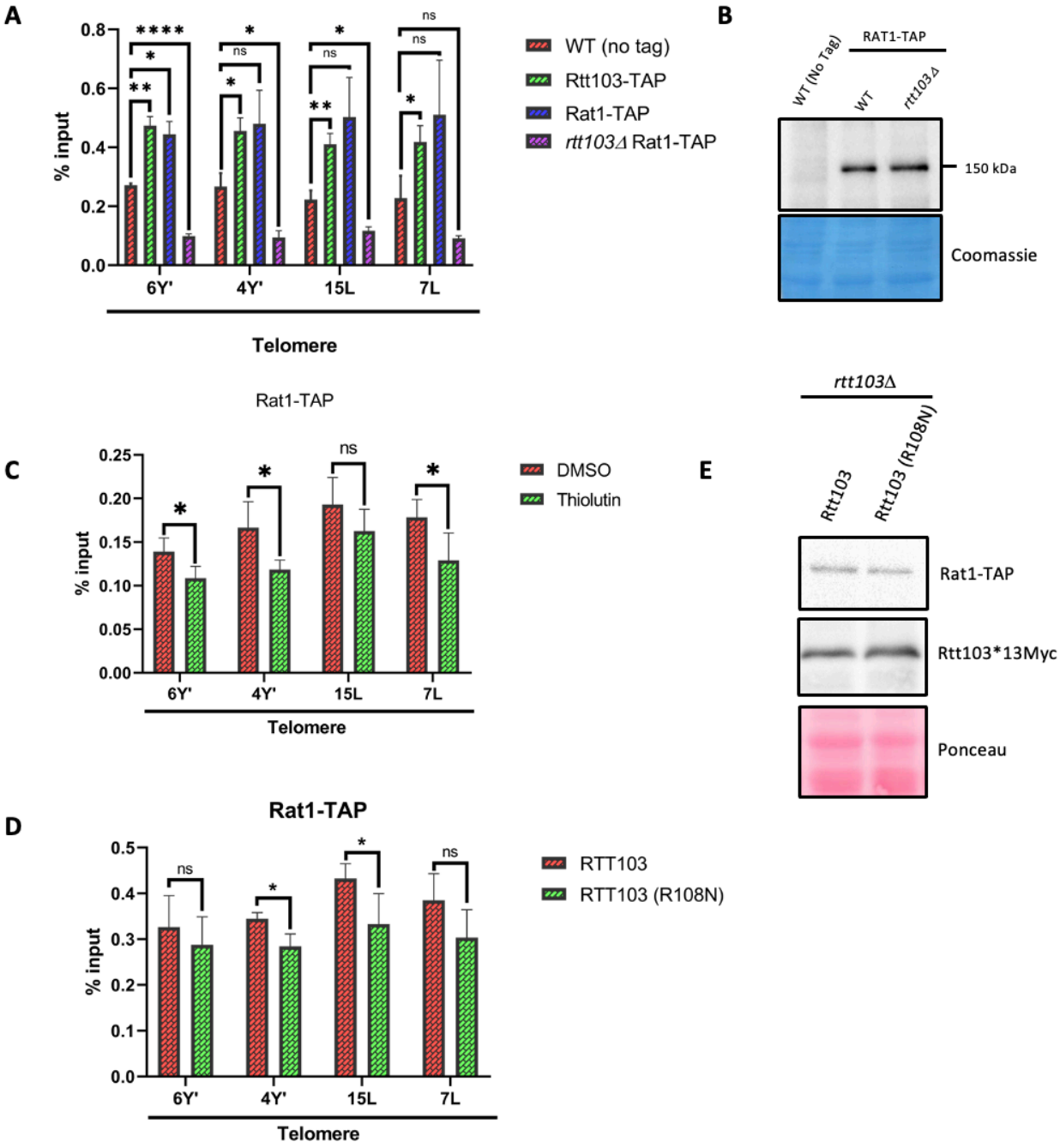

Figure 3.8: **Rtt103p recruits Rat1p to telomeres in a transcription-dependent manner:** **A.** ChIP using IgG sepharose was performed with the indicated strains encoding TAP tags. Enrichment of Rtt103 and Rat1 at the indicated telomeres was analysed. The WT without tag was used as the background control. The experiments were done three times, and the error bar represents the standard deviation. Statistical significance was calculated using two-way ANOVA (*p<0.05).

B. Western blot analysis of expression of Rat1-TAP in WT and *rtt103Δ*. Rat1 protein levels remain comparable to WT in *rtt103Δ*. The blot was stained with Coomassie after developing as a loading control.

C. ChIP was performed for Rat1-TAP in the presence and absence of thiolutin. Enrichment at the TERRA/Y′ loci was measured via qPCR and mean values were compared to their respective input. The experiment was done in triplicates and the mean was plotted. The error bars represent the standard deviation of mean and statistical significance was obtained using two-tailed Student's t-test (*p<0.05).

D. ChIP was performed for Rat1-TAP in *rtt103Δ* harbouring ectopically expressed WT *RTT103* or the point mutant *RTT103 (R108N).* Enrichment was measured via qPCR and mean values were compared to their respective input. All data are depicted as mean + SEM; n=3. P values were obtained from two two-tailed Student's t-test (*p<0.05). E. Western blot analysis to assess the expression of Rat1-TAP in *rtt103Δ* harbouring *Rtt103 (R108N).* Ponceau-stained blot serves as the loading control.

3.5 Discussion

In this work, we demonstrate that Rtt103, a transcription termination factor, is required for efficient silencing of telomeric and sub-telomeric transcripts. Similar to their role in transcription termination, both Rtt103 partners, Rai1 and Rat1, are also involved in regulating the levels of these transcripts. In *rtt103Δ*, silencing at the sub-telomeres is compromised at both modified and natural telomeres (Figure 3.3 and 3.4). All the three transcription termination mutants revealed varying degrees of silencing defects for the sub-telomeric genes, Y′ helicases and TERRA (Figure 3.5). Increasing evidence implying that Y′ helicases and TERRA transcripts are used as templates in telomerase-negative cells makes us speculate that this termination complex may have an important role to play in regulating telomere length in the absence of telomerase[113,130].

Although they work together in maintaining the levels of sub-telomeric transcripts, the variation in silencing between the three proteins might be due to impaired recruitment of Rat1 to the telomeres in case of *rtt103Δ* (Figure 3.8A), whereas in an *rai1Δ* mutant, it might stem from the compromised 5′-3′ exonuclease activity exhibited by Rat1, as it has been reported that Rai1 enhances Rat1 activity in vitro[54]. In addition, Rai1 possesses a decapping,

pyrophosphorylase, and exonuclease activity and recognizes unmethylated Gppp caps[54,139–141]. We speculate that some of the transcripts may be degraded by the activity of Rai1 in a co-transcriptional manner before the RNA is completely capped and protected. This could also be why *rai1Δ* has increased TERRA compared to both *rtt103Δ* and *rat1–1*. In addition, decapping by Rai1 would expose a 5′-phosphate that would make TERRA a substrate for Rat1 exonuclease activity.

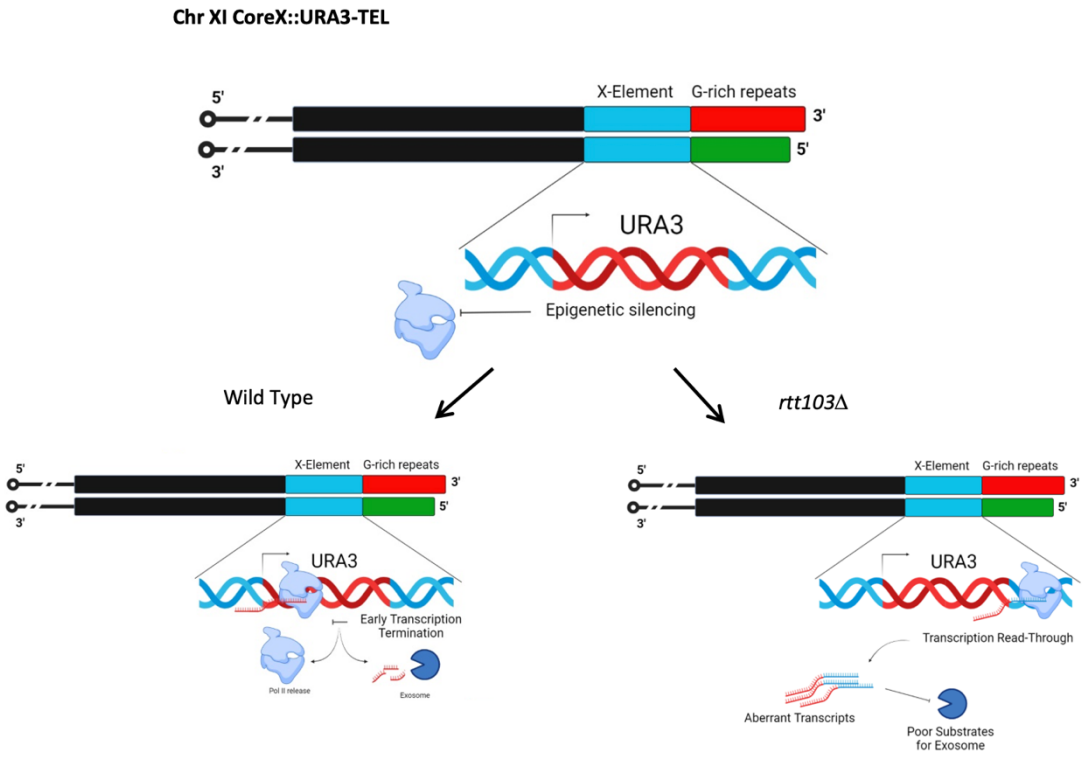

Figure 3.9: **Silencing at X-element heterochromatic loci:** In the wild type, the heterochromatic loci are kept repressed via three independent mechanisms: 1. Epigenetic silencing – Heterochromatin formation and repression of RNA Pol II Via SIR complex. 2. Premature termination of transcription and RNA Pol II recycling. 3. Any leaky transcripts will be targeted for exosome mediated degradation. In *rtt103*Δ, we speculate that it could be transcription read-through due to impaired recruitment of Rai1 and Rat1 for proper termination. As the aberrant transcripts are poor substrates for exosome mediated degradation, they exhibit increased stability in *rtt103*Δ.

In *rtt103Δ*, the stability of sub-telomeric transcripts is specifically altered, implying that these transcripts might be co-transcriptionally regulated in a Rat1-dependent manner (Figure 3.6A and B). Herein, we report that the enrichment of Rat1 at the telomeres is via Rtt103 in a transcription-dependent manner (Figure 3.8C and D). Previous studies have reported that when a telomere gets shortened, the association of Rat1 at the telomere is abolished and the continued presence of Rif1 and Rif2 is required for the association[113]. The absence of Rat1 at the telomeres in *rif1Δ* and *rif2Δ* could be due to the increased TPE and hence inaccessibility to the transcription machinery[142,143]. Alternately, Rat1 could be recruited independently by both Rap1/Rif1/Rif2 and Rtt103 to telomeres. It is known that TERRA exists in three different fractions, namely, chromatin-associated, nucleoplasmic, and cytoplasmic fraction[144]. It has been suggested that the non-chromatin associated fraction is regulated via degradation by Rat1[113]. Here in this study, we report the regulation of TERRA levels by the Rtt103-Rai1-Rat1 termination complex and propose a mechanism by which Rat1 may be targeted to the TERRA RNA.

How might Rtt103 regulate sub-telomeric transcript levels? We envision multiple possibilities (Figure 3.9 and 3.10). At the X elements where Sir-dependent silencing is robust, we propose that it is the escape transcription that is regulated by this complex as proposed earlier[145]. Normally, Sirp-dependent epigenetic silencing represses much of the transcription; any transcription that is initiated is prematurely terminated, and if transcription is completed then it is subjected to exosomal degradation. We suggest that the improper termination of transcription at the inserted *URA3* locus leads to production of transcripts with longer 3′-ends that might be poor substrates for exosomes, leading to export of this transcript and translation. At the non-coding TERRA site, one possibility is that the co-transcriptional recruitment of Rat1 (via Rtt103) to the sub-telomeric transcripts leads to degradation. As Rat1 can only act on uncapped 5′-ends, we think that once the RNA is capped, it has to be decapped and then Rat1 can degrade it. This is possibly post-transcriptional. Alternately or additionally, Rai1 could also independently exhibit this activity on nascent transcripts co-transcriptionally once recruited to the transcribing polymerase as it possesses a decapping and exonuclease activity on unmethylated 5′-caps[54,139–141]. As loss of Trf4, which is involved in targeting RNA to nuclear exosome, also increases TERRA abundance (albeit a minor one), it is possible that at least some of the TERRA is targeted to the nuclear exosome. We suggest that the TERRA transcripts produced in the absence of Rtt103-Rai1-Rat1 may have abnormal 3′-ends and may not be

degraded by the exosome machinery efficiently, thereby leading to an increased accumulation of these transcripts[146]. In sum, we propose that free TERRA is kept at very low levels in wild type by the combined action of Rtt103, Rai1, and Rat1 by targeting it in a co-transcriptional manner.

Interestingly, Rtt103 was initially isolated in a screen for mutants that revealed elevated Ty1 transcription and it was demonstrated that there was a moderate increase in Ty1 transcripts in *rtt103Δ*[44]. This raises the intriguing possibility that Rtt103 could be involved in negatively regulating Ty1 transcripts and in its absence, Ty1 transcripts are stabilized leading to increased cDNA and increased transposition. In another possible link, Y′ helicase transcripts are also higher in *rtt103Δ* and it is known that Y′ helicase is incorporated into the viral like particles produced in the Ty1 transposition cycle[132]. Together, these observations suggest a potential role for Rtt103-mediated RNA stability in processes affecting transposition and thereby genome stability. Of note, we show that Rtt103 could recruit Rat1, the exonuclease that has been implicated in the degradation of these transcripts. We show that association of Rtt103 with RNA Pol II is required for regulating the levels of transcripts and that this association is likely mediated via interaction of the C terminal domain of RNA polymerase with Rtt103.

Recent high-throughput transcriptional analyses have provided compelling evidence that transcription of telomeres and sub-telomeres is a conserved phenomenon observed across different phyla[104,114,116,134,147,148]. In humans, the TERRA length varies from few hundred to around 9 kb in size; it is transcribed in a centromere to telomere orientation and most of the population is 7-methylguanosine (m7G) capped at the 5′-ends while only 7% is polyadenylated[114]. In yeast, TERRA is transcribed from both Y′ and X-only containing telomeres[111]. Their average size ranges from ∼100–1200 bases and all are polyadenylated, while 5′-m7G cap has not been demonstrated directly[110]. The TERRA transcripts mostly originate in the sub-telomeres suggesting a defined transcription start site.

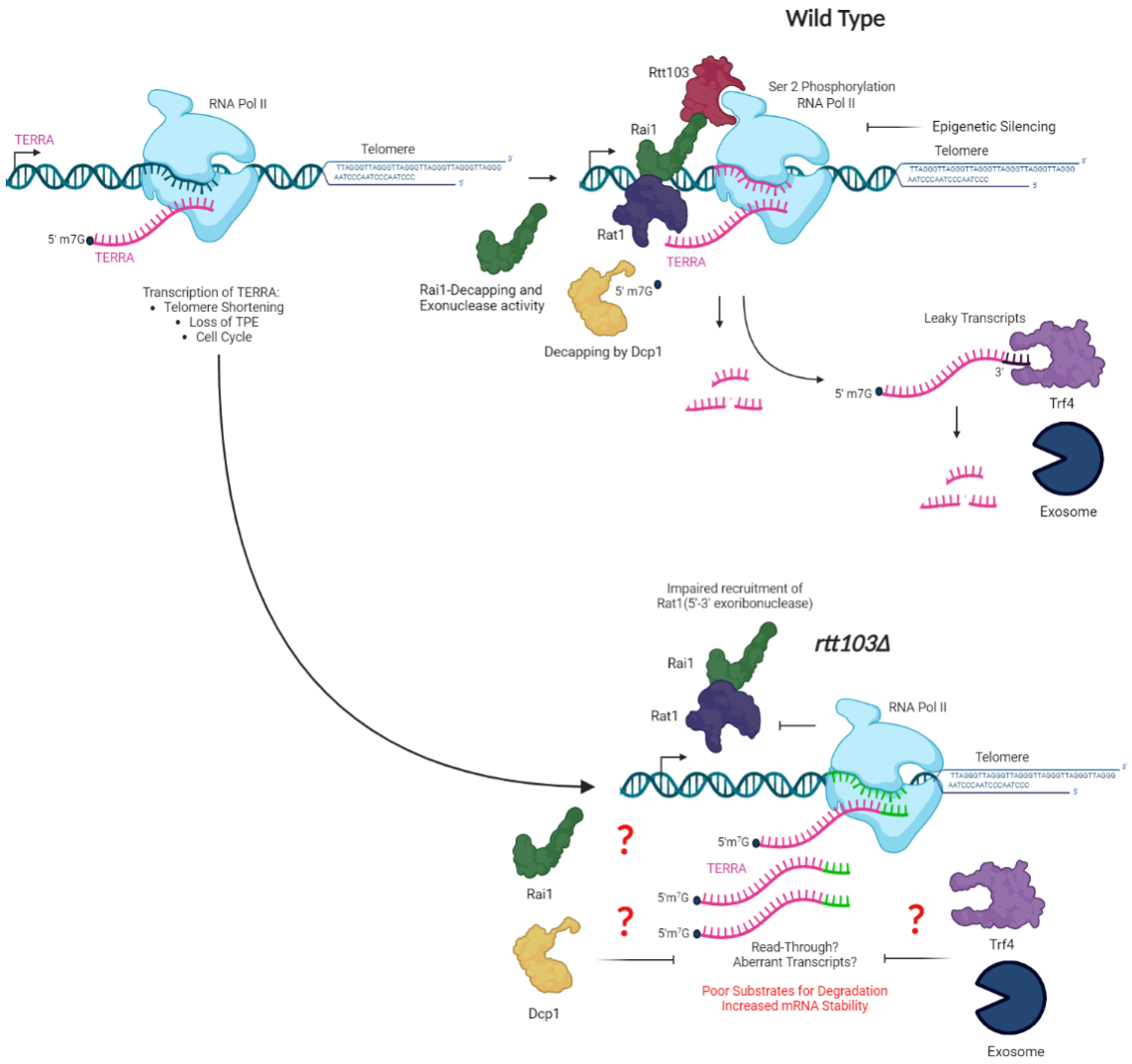

Figure 3.10: **Co-Transcriptional regulation of TERRA:** In the wild type, the TERRA levels are kept repressed at the epigenetic level via Rif1/2, Rap1, and Sir2/3/4 complex as well as post-transcriptionally via TRAMP mediated exosome degradation. In our study, we speculate that it might be co-transcriptionally regulated by the Rtt103-Rai1-Rat1 complex. Rtt103 recruits Rai1 and Rat1 in a transcription-dependent manner where Rai1 exhibits its pyrophosphohydrolyase activity towards mRNA having 5′-end cap and preferentially aids the substrate for Rat1 mediated exonuclease activity. In the absence of Rtt103, it leads to impaired recruitment of Rai1 and Rat1 resulting in termination defects and production of aberrant transcripts that are poor substrates for exosome mediated degradation.

In humans, CpG islands on a sub-set of telomeres appear to be promoters and cytosine methylation at these sites negatively regulates transcription[105,134]. As there appears to be defined initiation sites, the heterogeneity of TERRA size might be due to differential termination or processing of 3′-ends. TERRA molecules lack the conserved polyadenylation and cleavage signal 5′-AAUAAA-3′; in addition, the mechanism of transcription termination remains elusive. In general, protein-coding genes that contain the polyadenylation signal are terminated by the Rtt103-Rai1-Rat1 complex. However, for TERRA, our work suggests that the Rtt103-Rai1-Rat1 complex regulates stability, and we speculate that the termination mechanism may also be similar to that of protein-coding genes.

In summary, here we report a novel role for the transcription termination complex (Rtt103-Rai1-Rat1) in regulating the abundance of the sub-telomeric transcripts in a transcription-dependent manner. We show that the Rtt103 mutants have elevated levels of TERRA and other sub-telomeric transcripts that are usually silenced. Our findings suggest that Rtt103 potentially recruits the exonuclease, Rat1 in an RNA pol II-dependent manner to degrade these transcripts and regulate their levels in the cell. In the following chapter, we attempted to unravel the molecular mechanism of Rtt103 in DNA repair.

CHAPTER 4

Elucidating the molecular mechanism of Rtt103 in DNA repair

4.1 Introduction

DNA damage response (DDR) is a repository of pathways, each of which is selectively regulated according to the type of lesion and/or the cell cycle stage[149]. DNA double-strand breaks are generally repaired via two significant pathways, namely NHEJ (Non-Homologous End Joining) and HR (Homologous Recombination). NHEJ is an error-prone process and is favored in G0/G1 phase of the cell cycle in yeast, whereas HR is accurate and favored during the S and G2 phases of the cell cycle, where the homologous template is available in the form of sister chromatids[150]. In an earlier study from our laboratory, we reported that overexpression of Rtt103 partially suppresses the MMS sensitivity and *ts* phenotype exhibited by *yku70*Δ[81]. Yku70 is a well-conserved protein with a predominant role in the Non-Homologous End Joining (NHEJ) repair pathway. Further, the double mutants *rtt103*Δ *yku70*Δ revealed an exacerbated sensitivity to MMS indicating that they might function in two independent pathways to maintain genome integrity. Alongside, we have also found that *rtt103*Δ mutants are sensitive to a wide range of DNA damaging agents which introduce multiple genomic insults (MMS/ UV/ EcoRI endonuclease) or regulated single DSB at a specific locus (SceI or HO endonuclease)[81].

Several other genome-wide studies also indirectly indicate that loss of *rtt103*Δ results in increased basal DSB and genome instability, including MMS sensitivity, DNA:RNA hybrid formation, increased Rad52 foci, genetic interaction with DNA repair proteins, IR sensitivity, chromosomal rearrangements, and delayed repair kinetics[78,79,81,151–154]. Although Rtt103 was co-purified with Rai1 and Rat1, none of the above studies identified Rat1 or Rai1 to have a role in genome stability. Further, the mammalian counterpart CREPT/RPRD1B participates in DNA repair, whereas RPRD1A (paralog) mainly associates with RNA Pol II and is involved in the regulation of transcription termination[43]. In mammals, it associates with NHEJ factors Ku70, Ku86, and Artemis and also forms additional complexes with DNA mismatch repair proteins

MLH2, PMS2, and MSH2[78,79]. It regulates HR pathway via transcriptional regulation of CDK1- a major effector involved in the regulation of DNA damage response[155].

Despite these interactions and indications of a role in genome stability, the mechanistic role of Rtt103 and the pathway it regulates primarily remains undeciphered. Therefore, we took multiple approaches to address this question. First, we asked whether transcriptional machinery is critical for its function in DNA repair. Next, we performed yeast 2-hybrid screens to obtain potential interaction partners. Finally, we performed RNA sequencing of *rtt103Δ* to see if some specific transcripts involved in genome stability were differentially regulated.

4.2 Results

Is Rtt103 interaction with RNA polymerase II crucial for its function in DNA Repair?

In all eukaryotes, the largest subunit of RNA pol II harbours a highly conserved C-terminal domain (CTD), which is composed of multiple heptapeptide repeats with the consensus sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 (Y_1-S_2-P_3-T_4-S_5-P_6-S_7)[156]. While the number of repeats varies from species to species, the CTD of budding yeast contains 26 repeats whereas in mammals it has 52 repeats; the amino acid sequence is conserved throughout evolution[156]. The CTD is essential for the function of RNA Pol II. Based upon the phosphorylation status of this heptapeptide repeat, various protein complexes associate with RNA Pol II during transcription and regulate initiation, elongation, termination of transcription, and post-transcriptional RNA processing[6,157,158]. Based on recent genome-wide studies, the typical pattern of RNA polymerase phosphorylation for protein-coding genes is that phosphorylation of serine 5 (Ser5-P) and Serine 7 (Ser7-P) dominates at the 5'-end, whereas Serine 2 phosphorylation (Ser2-P) is enriched at the 3'-end[32,158]. Rtt103 interacts with the Ser2-P RNA Pol II and is proposed to aid the recruitment of Rai1 and Rat1[47]. To delineate Rtt103 residues involved in its interaction with RNA Pol II Lunde *et al* , created a point mutant *RTT103 (R108N)* and showed approximately 20%-50% reduction in the interaction between RNA Pol II and Rtt103[138]. Given this information, *RTT103 (R108N)* and *CIDΔ RTT103* strains have been constructed to check whether interaction of Rtt103 with RNA Pol II is crucial for DNA repair.

Effect of Rtt103 (R108N) on DNA repair

To assess whether transcription is necessary for Rtt103 to reach the DSB site, a point mutation that replaces arginine with asparagine *(R108N)* in the CID domain of Rtt103 was introduced. This point mutation was earlier shown to reduce the interaction of Rtt103 with the CTD RNA Pol II by about 50%[138]. The mutation was introduced by PCR into Rtt103-13xMyc, confirmed via sequencing and expression was confirmed using antibodies against the myc epitope. The reduced interaction with RNA Pol II was confirmed via yeast two-hybrid and β-gal assays (Figure 4.1B). The MMS sensitivity of this point mutation was assessed first. *rtt103*Δ strain was transformed with plasmid encoding either wild type Rtt103 or the point mutant and was tested for growth on plates containing MMS. Wild type and *rtt103*Δ transformed with empty vector were used as controls.

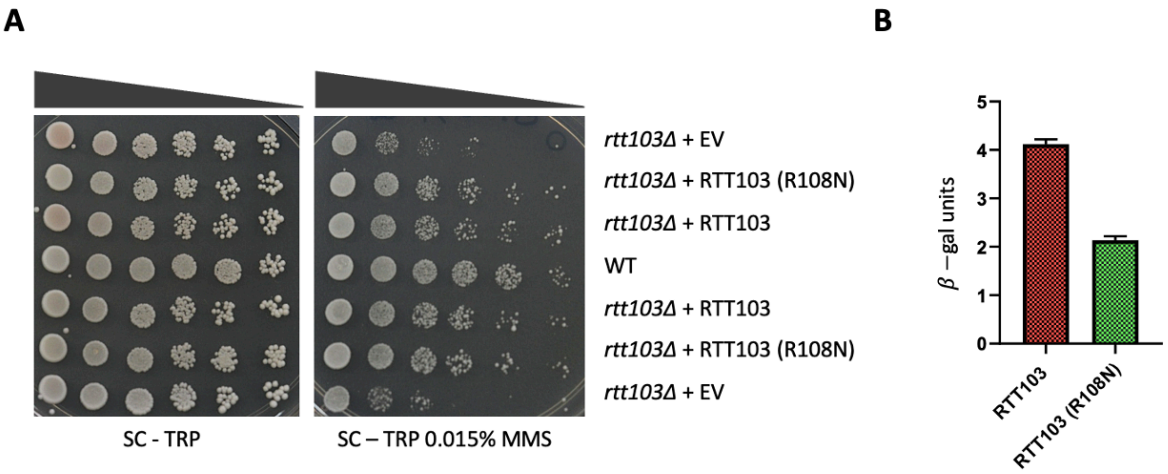

Figure 4.1: **Effect of RTT103 (R108N) on DNA repair:** A. WT and *rtt103*Δ strains were transformed with either *RTT103*, *RTT103(R108N)*, or E-Vector (2μ) plasmids. The transformants were initially grown in SC-TRP broth and 5 μl of 10-fold serial dilution was spotted on SC-TRP and SC-TRP + 0.015% MMS plates. B. The β-gal activity of yeast cells co-expressing either RTT103-BD/ RTT103 (R108N)-BD and RNA Pol II CTD-AD fusions.

As seen in Figure 4.1A, WT cells with empty vector and *RTT103* over-expression grew normally on the plate containing 0.015% MMS, whereas *rtt103*Δ cells were sensitive to MMS. When *RTT103(R108N)* was expressed in *rtt103*Δ, it was observed that the repair phenotype was complemented similar to that of *RTT103* expression. This shows that

either Rtt103 interaction with RNA Pol II is not necessary for genome stability function of Rtt103 or that the reduced interaction with RNA Pol II is sufficient for the function of Rtt103.

To distinguish between these two possibilities, a *CIDΔ RTT103,* which is entirely devoid of the interacting domain, was constructed and tested for sensitivity.

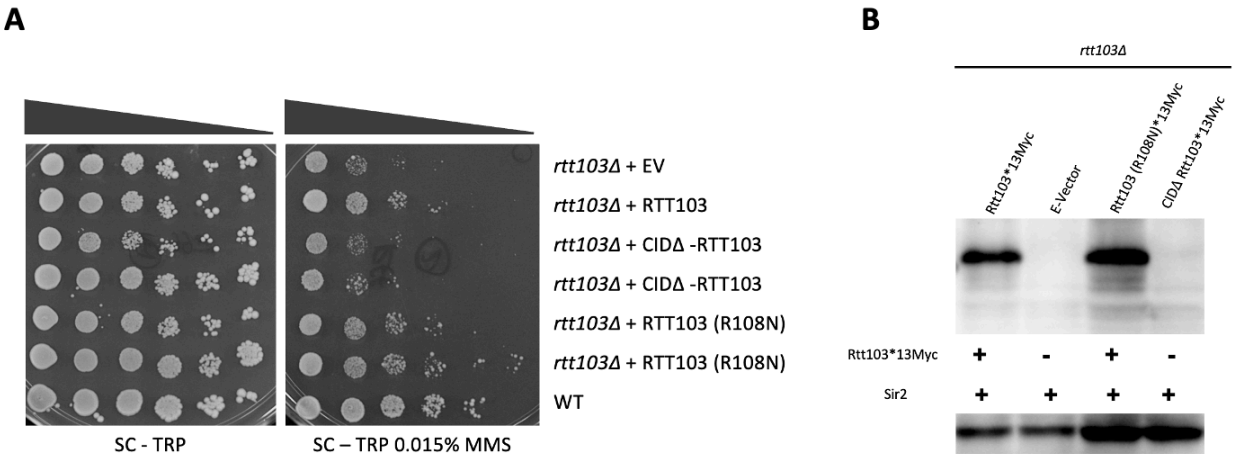

Figure 4.2: **A. Effect of CIDΔ Rtt103 (R108N) on DNA repair:** WT and *rtt103*Δ strains were transformed with either RTT103, CIDΔ RTT103, RTT103(R108N), or E-Vector (2μ) plasmids. The transformants were initially grown in SC-TRP broth and 5 μl of 10-fold serial dilution was spotted on SC-TRP and SC-TRP + 0.015% MMS plates. **B. CID domain of RTT103 is necessary for its stabilization:** *rtt103*Δ strain was transformed with either RTT103, RTT103 (R108N), CIDΔ RTT103, or E-Vector (2μ) plasmids. Proteins were harvested from cells and separated by SDS-PAGE and monitored for Rtt103 *13myc by western blot. Sir2 serves as the loading control.

As seen in Figure 4.2A, WT cells with empty vector and *rtt103*Δ strain carrying plasmid encoding either RTT103-13xmyc / RTT103 (R108N)-13xmyc grew normally on the plate containing 0.015% MMS. No growth was observed in *RTT103*Δ*CID* on plates containing MMS. *RTT103*Δ*CID* cells could not complement the DNA repair phenotype on MMS plates. We assessed the functionality of the construct by sequencing and expression analysis via western blotting. Although the *RTT103*Δ*CID* construct was confirmed via sequencing, the expression of *RTT103*Δ*CID* remained undetectable via western blotting. Sir2 protein levels

served as loading controls (Figure 4.2B). This suggests that the CID domain of Rtt103 is possibly necessary for its stability.

Construction of Tetracycline-regulatable expression system to study DSB repair in detail during active transcription

As the previous experiments could not establish whether the association with RNA polymerase were necessary for Rtt103 function in genome stability, we set out to directly test if transcription was necessary for Rtt103 function. In our earlier work from the lab, we have shown that *rtt103*Δ mutants were defective in repairing a single break introduced at a specific locus[81]. However, if transcription of the region containing the DNA break was required for this process was not tested. These experiments were done in a strain carrying *URA3* flanked by sites for the enzyme SceI. When SceI was expressed, it introduced 2 double-stranded breaks, one on either side of *URA3* coding sequence. In order to assess whether transcription was required for repair of these double-stranded breaks, we created a transcription inducible *URA3* locus by replacing the endogenous *URA3* promoter with a tetracycline (Tet)-regulatable promoter with a single SceI site downstream of the stop codon (see figure 4.3).

The Tet-regulatable expression system can be of two types, namely TET-ON or TET-OFF system. The principle behind tetracycline repressible promoter is that the Tet repressor (tetR) and trans-activator (tTA) fusion proteins bind at promoters containing Tet-O DNA sequences only in the absence of tetracycline or its analogue doxycycline and promote transcription. The addition of the drug displaces the fusion protein and represses transcription. For tight control and a better dynamic range of regulated transcription, the Tet-OFF system was constructed by replacing the *URA3* promoter via PCR-mediated homology recombination. The sequence that encodes SceI endonuclease was inserted at the *lys2* locus, and its expression could be induced via galactose. A schematic illustration of the locus is depicted in Figure 4.3.

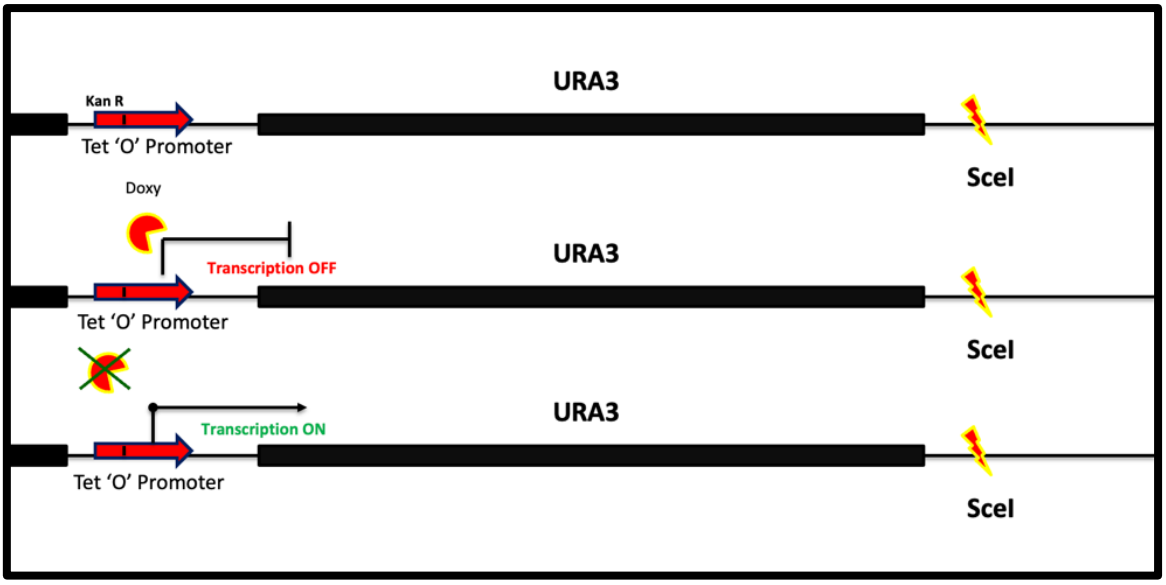

Figure 4.3: Schematic illustration of KanMX4-tetO$_p$-*URA3* locus showing I-SceI site at the 3′-end. In the presence of doxycycline, transcription is shut off, whereas in the absence of doxycycline, the promoter can be transcribed.

In general, wild-type cells can repair the I-SceI endonuclease-mediated breaks and form colonies upon continuous induction. The repair mechanism usually involves NHEJ with a loss of couple of nucleotides and which results in the loss of enzyme recognition site and becomes resistant to further enzymatic action. The strain which cannot repair the breaks will undergo cell cycle arrest and cannot form colonies on galactose plates. In the presence of doxycycline and galactose, the I-SceI-endonuclease-mediated break is on and transcription is off. In the presence of galactose without doxycycline, both I-SceI endonuclease-mediated breaks and transcription is on. The tight regulation of *URA3* was validated via both colony forming assays and q-RT-PCR in the presence of doxycycline (data not shown).

Relative survival assay

Colony-forming assays on galactose plates (and glucose plates as control), both in the presence and absence of doxycycline, were performed using the *rtt103*Δ tet-O$_p$-*URA3* strains. As seen in Figure 4.4A, the presence of doxycycline altered the repair efficiency of I-SceI endonuclease-treated cells; the repair was more efficient in the absence of doxycycline when

transcription was on. This was seen in both wild type and *rtt103*Δ. This is consistent with previous studies that have shown that transcription at the break sites improves the efficiency of DNA repair[159]. *rtt103*Δ strain did show reduced survival compared to wild-type, but the transcription status didn't have any significant reduction in the overall survival rate of *rtt103*Δ in comparison to the wild type (Figure 4.4A and B). This suggests that while transcription improved the repair of DNA breaks, Rtt103 does not appear to have any role in this repair enhancement.

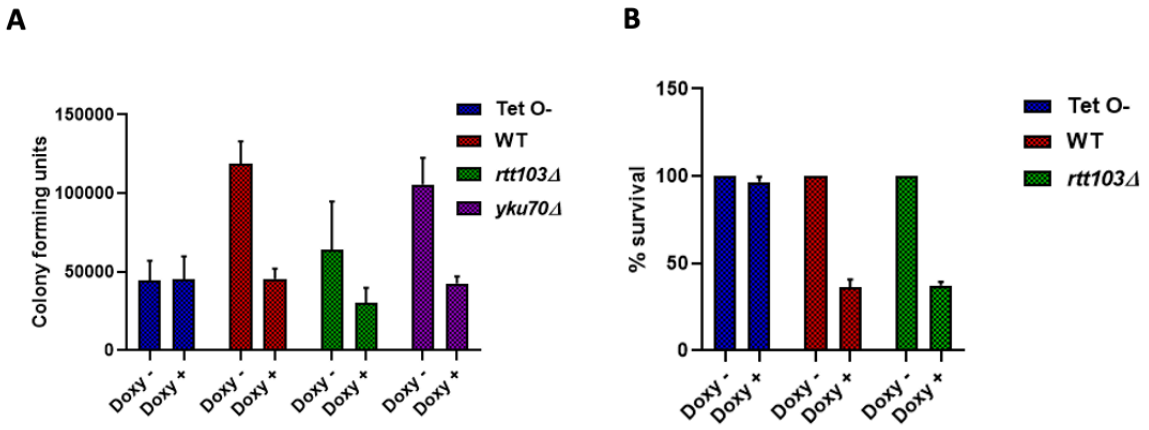

Figure 4.4: **Transcription enhances DNA repair:** A. The colony forming units of WT, *rtt103*Δ, and *yku70*Δ with and without doxycycline when the DNA break was induced is plotted. A strain without any modification was used as the control (Tet O^-). All data are depicted as mean + SEM; n=3. B. Bar graphs representing the percent survival of WT, *rtt103*Δ, and Tet O^-.

Isolation of interacting partners of Rtt103 to understand the molecular role of Rtt103 in DNA damage response

The yeast two-hybrid (Y2H) system is a powerful tool for the identification of binary transient protein-protein interactions (PPI); it can be applied in a high-throughput manner to detect interactions across the entire proteome of an organism. To identify interacting partners of Rtt103 as a means to obtain insights into the mechanism of Rtt103 function in genome

stability, we performed yeast two-hybrid screen. To detect the proteins interacting with Rtt103, Gal-BD fusion of Rtt103 was made and self-activation was tested. We further functionally validated the constructs for DNA repair complementation activity via MMS assay (data not shown).

The first round of Y2H screen was initiated with full length F-RTT103 against the genomic GAD library as described by Gietz and Woods[85]. A total of 11 positive transformants were obtained in the initial screen. Plasmids were isolated from all the 11 positive interactors. These were re-tested for interaction with Rtt103 and were also subjected to restriction enzyme analysis to check whether these were all different. Seven plasmids produced unique digestion patterns, and hence, those 7 plasmids were sequenced to identify the interacting partner.

Among the 7 positive interactors, only one was found to be strong interactor i.e., ADE+ and all the remaining were HIS+. Though more than 30 interactors have been reported for Rtt103 in EMBL-EBI IntACT via tandem affinity purification, we hoped to identify an interactor which might shed some light on how Rtt103 affects the genome stability. From the primary screen, we did not obtain any significant interactor known to be involved in DNA repair.

Hence, a second screen was attempted with a slight modification in the protocol. In order to elicit the highly coordinated cascade of events involved in DDR, the cells were grown with 0.03% MMS (sub-lethal dose) and high efficiency transformation was performed. In this screen, we obtained 381 positive interactors [(His+)-374/(Ade+)-28]. Initially, ADE+ colonies alone were subjected for further processing. After pGAD enrichment and restriction pattern analysis, plasmids were subjected to sequencing.

A total of 29 unique interactors were obtained as interactors for Rtt103 in these two screens. They were functionally categorized broadly fell under RNA Processing (Fir1, Scp160), DNA Repair (Wss1, Yen1), regulators of RNA polymerases (Srp1, Kns1, Cad1), proteins in the sumoylation pathway (Uls1, Wss1, Nfi1), transcription Factors (Mig1, Hap4), endoplasmic reticulum associated proteins (Srp101, YBR137W, Ufd1, Srp72) and a few others as listed in Table 4.5.

Gene	Interacting Coordinates	Function	No of times obtained
FIR1/YER032W (2631bp)	1815-2631bp 153-2631bp 721-2631bp	Protein involved in 3' mRNA processing and regulation of cytokinesis	7 2 1
SCP160/YJL080C (3669bp)	2637-3382bp	polysome-associated mRNA-binding protein/ Involved in mRNA trafficking (similar to vertebrate vigilins)	1
RPO21/YDL140C (5202bp)	4314-5202bp	RNA pol II largest subunit B220 (heptapeptide domain)	1
NFI1/YOR156C (2181bp)	1042-1606bp	SUMO E3 ligase	1
ULS1/YOR191W (4860bp)	995-1732bp	Swi2/Snf2-related translocase, SUMO-Targeted Ubiquitin Ligase (STUbL); required for maintenance of NHEJ inhibition at telomeres	1
TEA1/YOR337W (2280bp)	1-693bp	Ty1 enhancer activator	2
ATG4/YNL223W (1485bp)	962-1065bp	Conserved cysteine protease required for autophagy	1
WSS1/YHR134W (810bp)	678-810bp	SUMO-ligase and SUMO-targeted metalloprotease/ Involved in DNA repair	3
YEN1/YER041W (2280bp)	1508-2280bp	Holliday junction resolvase	4

HAP4/YKL109W (1665bp)	1-436bp	Transcription factor/ glucose-repressed Hap2p/3p/4p/5p CCAAT-binding complex	1
MIG1/YGL035C (1515bp)	12-590bp	Sequence-specific DNA binding transcription factor involved in glucose repression	2
TAT1/YBR069C (1860bp)	204-302	Amino acid transporter for valine, leucine, isoleucine, and tyrosine/low-affinity transporter for tryptophan and histidine	1
NIS1/YNL078W (1224bp)	1051-1224bp	SUMO-binding protein involved in axial bud site selection	2
LRE1/YCL051W (1752bp)	81- 476bp	Protein involved in regulation of cell wall integrity and hyperosmotic stress response	1
SRP1/YNL189W (1629bp)	122-1269bp	Karyopherin alpha homolog; forms a dimer with karyopherin beta Kap95p to mediate import of nuclear proteins, binds the nuclear localization signal of the substrate during import	1
KNS1/YLL019C (2214bp)	1-574bp	Protein kinase involved in negative regulation of Pol III transcription: effector kinase of the TOR signalling pathway and phosphorylates Rpc53p to regulate ribosome and tRNA biosynthesis	3
SAP1/YER047C (2694bp)	533-2694bp	Putative ATPase of the AAA family	1

SRP101/YDR292C (1866bp)	411-1305bp	Signal recognition particle (SRP) receptor alpha subunit: contain GTPase domains	1
YBR137W (540bp)	64-346bp	Protein with a role in ER delivery of tail-anchored membrane proteins.	1
YDR423C/CAD1 (1230bp)	1-742bp	AP-1-like basic leucine zipper (bZIP) transcriptional activator. RNA pol II binding (+ve regulator of transcription under stress)	1
SMM1/YNR015W (1155bp)	1-670bp	Dihydrouridine synthase (Modifies uridine residues at position 20 of cytoplasmic tRNAs)	2
CCC1/YLR220W (969bp)	723-969bp	Vacuolar Fe2+/Mn2+ transporter	1
VMA13/YPR036W (1437bp)	1-686bp	Subunit H of the V1 peripheral membrane domain of V-ATPase.	1
UFD1/YGR048W (1086bp)	859-1086bp 813-1086bp	Involved in regulated destruction of ER membrane proteins such as HMG-CoA reductase (Hmg1/2p) and cytoplasmic proteins (Fbp1p)	3 1
SRP72/YPL210C (1923bp)	1-375bp	Core component of the signal recognition particle (SRP)/ Targets nascent secretory proteins to the endoplasmic reticulum (ER) membrane	1
PHB1/YGR132C (1002bp)	774-864bp	Subunit of the prohibitin complex (Phb1p-Phb2p)	1

		(prohibitin is a 1.2 MDa ring-shaped inner mitochondrial membrane chaperone)	
GID7/YCL039W (2238bp)	1-484bp	Subunit of GID Complex (GID complex is involved in proteasome-dependent catabolite inactivation of fructose-1,6-bisphosphatase)	1
XDJ1/YLR090W (1380bp)	195-727bp	Chaperone with a role in facilitating mitochondrial protein import	1
YPR159C (102bp)	1-37bp	Hypothetical Protein	1

Table 4: List of interactors obtained for Rtt103 along with their matching co-ordinates and description

Global Gene Expression Analysis in *rtt103*Δ

DNA damage induces a wide variety of responses ranging from protein modification, sub-cellular translocation, differential protein stability, and induction of gene expression[160]. Besides the already reported ~150 proteins directly involved in DNA repair processes, there are numerous other proteins with diverse biological functions that play a crucial role in cellular recovery following DNA damage[161]. However, the mechanistic significance of many of these proteins in the context of cellular recovery is still not completely understood. *rtt103*Δ mutants are susceptible to various forms of genomic insults[81]. Besides, even under unperturbed conditions, increased basal levels of R-loops, Rad52 foci, γ-H2AX, and 53BP1 foci are seen in *rtt103*Δ and in the loss of function mutants of the human homologue[3-9]. This suggests that Rtt103 and its homolog are involved in genome protection. In order to unravel the specific contributions of Rtt103 and its mechanistic relevance in DNA damage, we attempted to measure the global transcriptomic change via RNA sequencing.

RNA-seq was performed by isolating RNA from two biological replicates of WT and *rtt103*Δ. After quality check, the RNA samples were handed over to Genotypic Technologies for further processing. The data obtained were analysed in the laboratory. The percentage of aligned reads was >85%, and the Spearman's correlation coefficient was >0.99 between the replicates. The differential expression analysis was done in comparison to the wild type via the empirical Bayes model (EBSeq-R). A target FDR of 0.01 was used, and the list of genes obtained was further refined using a minimum fold change cut-off of 2. The cut-off employed was >2-fold for up-regulation and <2-fold for down-regulation. Here, we report a total of about 255 genes (139 up-regulated and 116 down-regulated) were differentially regulated between WT and *rtt103*Δ mutant (Tables 4.9 and 4.10). Functional clustering and network analysis of significantly up-regulated and down-regulated gene categories was done using ClueGO V2.3.3. The results revealed surprising patterns of variation across a wide range of targets.

WT vs *rtt103*Δ down-regulated gene categories: A total of 116 down-regulated genes are listed in Table 4.10. The most notable group includes nucleotide and RNA metabolism, ribosomal biogenesis, and assembly factors (Figure 4.6A and B). The biogenesis of ribosomes involves several major steps: 1. Transcription of ribosomal RNA (rRNA) by RNA Pol I and III. 2. Processing and maturation of rRNA by coordinated action of various endo- and exo-nucleases. 3. Assembly and export of ribosomal subunits. 4. Final maturation and activation in the cytoplasm[162]. In *rtt103*Δ, ribosomal biogenesis is compromised at every step (RNA Pol I/Pol III subunits/35s Pre-rRNA/Ribosomal subunits/Ribosomal assembly factors and chaperones). We further functionally validated the expression for a select set of genes from multiple categories using qRT-PCR (Figure 4.8). We also extended the study to *rai1*Δ to assess whether the entire termination complex is involved in the regulation. Surprisingly, *rai1*Δ also displayed a similar expression profile for many of the genes tested. In sum, ribosome biogenesis was found to be compromised in both *rtt103*Δ and *rai1*Δ.

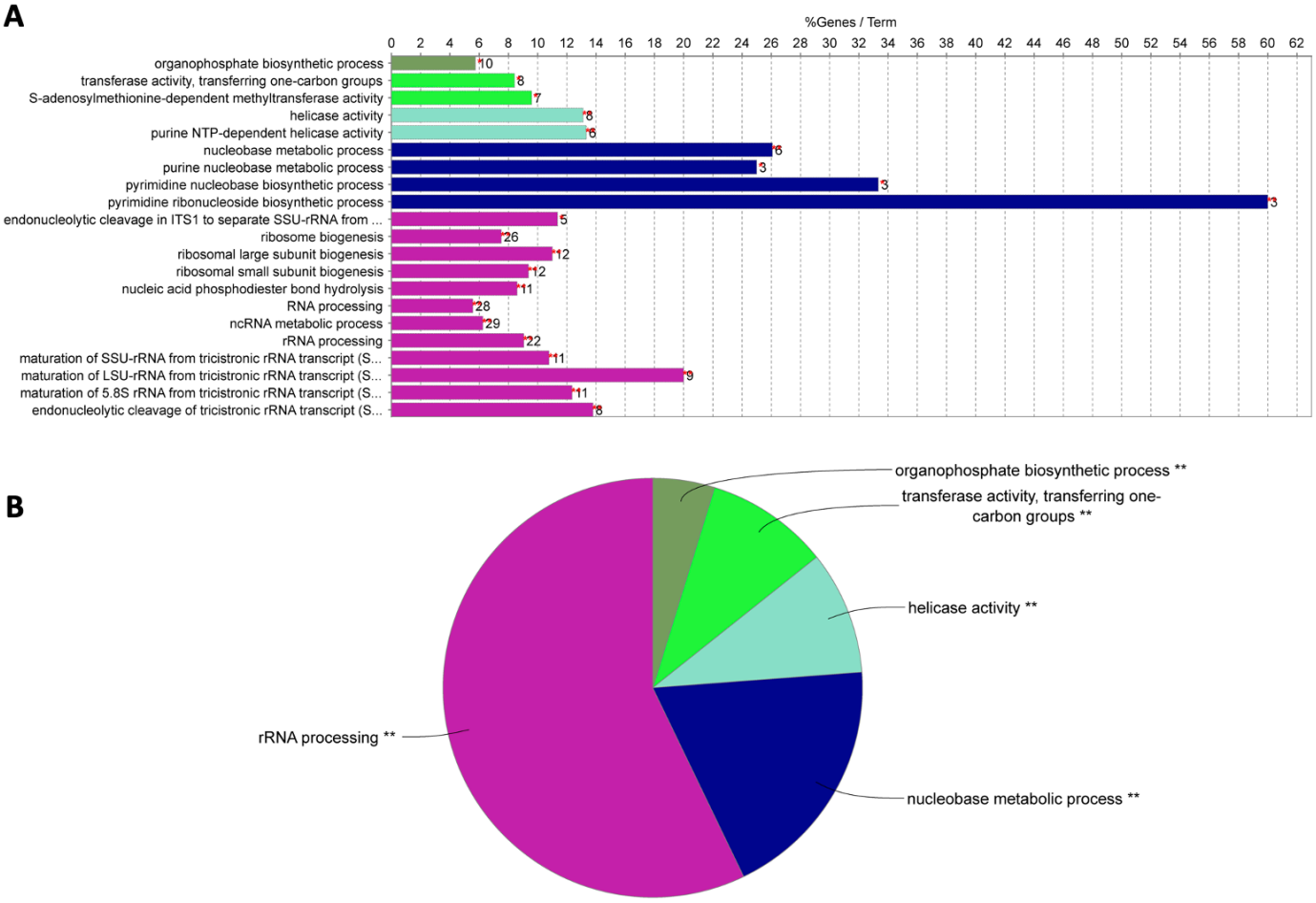

Figure 4.5: A. **GO ontology analysis for down-regulated genes:** The number of corresponding genes associated with each term are indicated. The percentage of genes associated with a specific term is listed on the bars. B. A functional group overview chart including specific terms for downregulated genes. The significance of the enriched GO term is denoted by *p < 0.05 and **p < 0.01.

WT vs *rtt103*Δ up-regulated gene categories: The 139 up-regulated genes are listed in Table 4.9. Functional clustering revealed that a significant number of genes related to mitochondrial biogenesis, energy metabolism, and mitochondrial ribosome biogenesis were up-regulated (Figure 4.7A and B). Cells invoke several compensatory mechanisms to ensure survival in compromised situations. The down-regulated gene categories in *rtt103*Δ and *rai1*Δ is a clear indication that de novo transcription and translation is compromised even under

unperturbed condition. Previous evidences suggest that when cytosolic protein synthesis is compromised, it has an inverse relationship with mitochondrial protein synthesis. Though this reciprocal relationship has been established as an adaptative mechanism for survival, the mechanism of compensation still remains elusive[163,164].

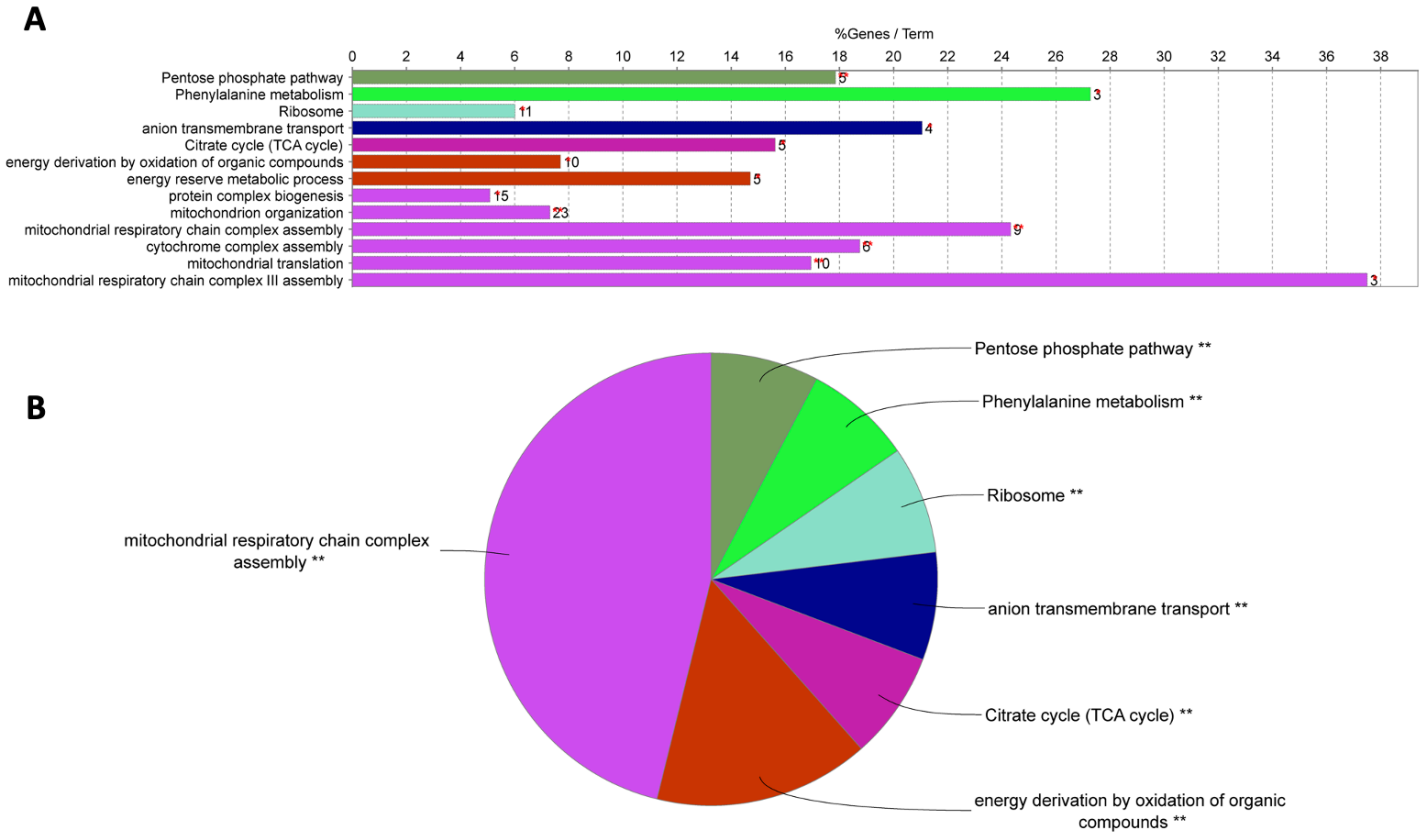

Figure 4.6: **A. GO ontology analysis for up-regulated genes:** The number of corresponding genes associated with each term is indicated. The percentage of genes associated with a specific term is listed on the bars. **B.** A functional group overview chart including specific terms for up-regulated genes. The significance of the enriched GO term is denoted by $*p < 0.05$ and $**p < 0.01$.

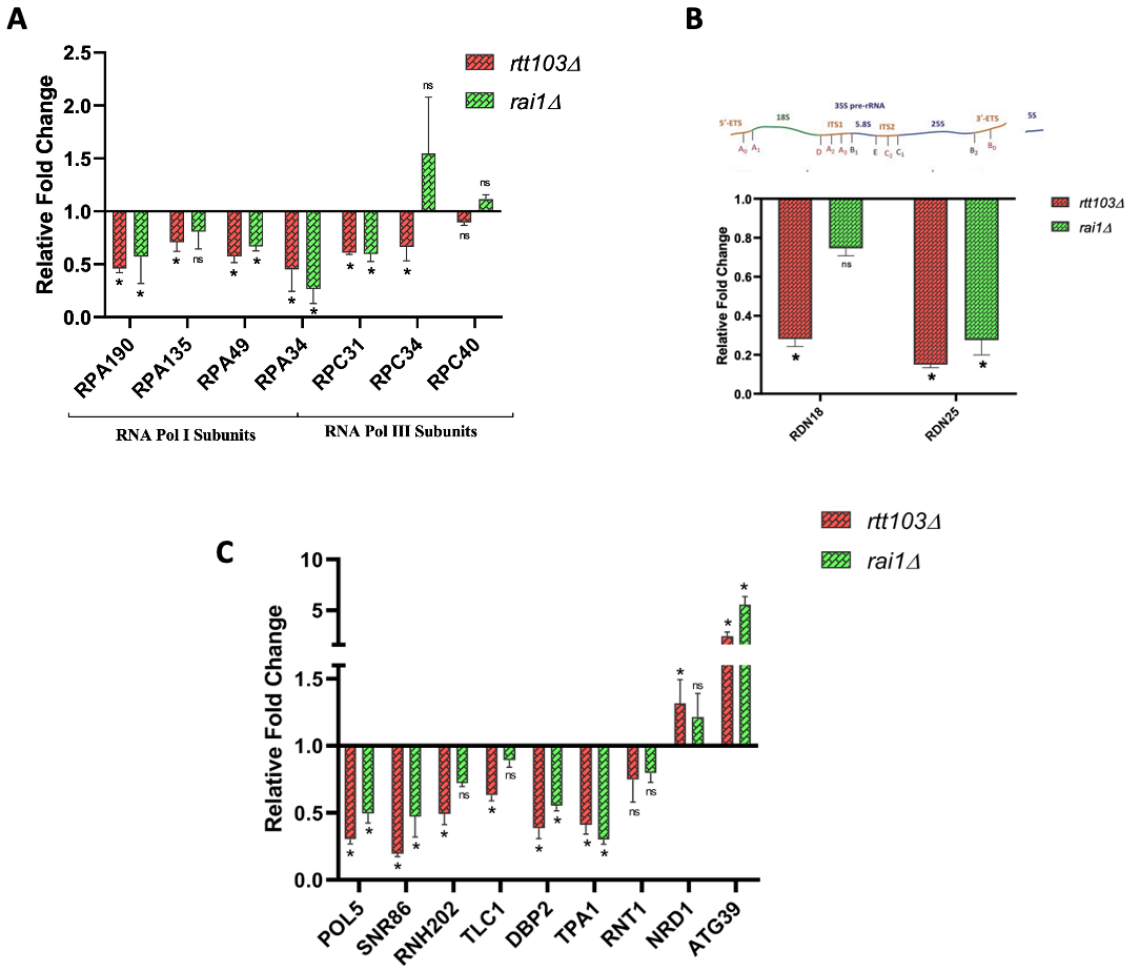

Figure 4.7: **A**. q**RT-PCR validation of RNA-seq data:** RNA was isolated from WT, *rtt103*Δ, and *rai1*Δ was converted to cDNA. cDNA was then subjected to qRT-PCR to assess the relative expression: **A.** RNA Pol I and RNA Pol III subunits. **B.** rRNA – RDN18, RDN25. C. various other classes of genes that showed significant change in RNA-Seq. All data are depicted as mean + SEM; n=3. p values were obtained from two-way ANOVA.

4.3 Discussion

As Rtt103 was found to be involved in DNA repair, we attempted to unravel the molecular mechanism underlying Rtt103 function. Our findings so far clearly indicate that Rtt103 interaction with RNA Pol II is not crucial for DNA repair. Transcription *per se* does not have a direct influence on *rtt103*Δ repair efficiency (Figure 4.1 and 4.4). The yeast two hybrid system yielded a total of 29 interactors for Rtt103. Among them, 27 were novel (Table 4.5). Among the interactors, Fir1 was the predominant hit obtained 10 times; however, very little is known about its role in the 3′-end formation of mRNA coding genes[165,166]. Recently, Fir1p has been implicated in cytokinesis[167]. In addition, we have found several other classes of interactors, including DNA Repair (Wss1, Yen1), proteins involved in sumoylation (Uls1, Wss1, Nfi1), transcription factors (Mig1, Hap4, Cad1), endoplasmic reticulum-associated proteins (Srp101, YBR137W, Ufd1, Srp72), mitochondrial proteins (Xdj1, Phb1), autophagy (Atg4), and various other miscellaneous (Tea1, Gid7, Tat1, Nis1, Smm1, YPR159C, Ccc1, Lre1).

In an actively dividing cell, the relative distribution of RNA is 80% rRNA, 15% tRNA, and 5% mRNA[168]. RNA Pol I is composed of 14 subunits and plays an essential role in the synthesis of the largest rRNA precursor. It accounts for 60% of the total transcription in the cell. RNA Pol III is composed of 17 subunits and is involved in the synthesis of tRNAs, 5S rRNAs, and other non-coding RNA[162,169,170]. From our RNA-Seq data, it was evident that *rtt103*Δ mutants displayed downregulation of several RNAP I (4) and RNAP III (3) subunits (Figure 4.6), whereas RNA Pol II levels remained unaltered in *rtt103*Δ. Previous studies, both in yeast and in higher eukaryotes, suggest that RNA polymerases are co-regulated under many circumstances[171,172]. Secondly, among the downregulated transcripts, more than 60% of the transcripts were observed to be involved in cytoplasmic ribosomal RNA processing (35s Pre-rRNA/Ribosomal subunits/ribosomal assembly factors and chaperones) (Figure 4.6). The exact mechanism of this coordinated repression of rRNA and ribosomal protein synthesis is not well understood. Furthermore, *rai1*Δ revealing similar expression profile for many of the tested genes suggests the role of this termination complex in the regulation of RNA Pol I, RNA Pol III, and ribosome biogenesis. In the following chapter, we have further investigated the connection between ribosome biogenesis and Rtt103.

WT vs *rtt103*Δ Up-regulated genes

Standard Name	Systematic Name	Description	Fold Change
HXT6	YDR343C	HeXose Transporter	1214.90
PHO84	YML123C	PHOsphate metabolism	17.24
SPL2	YHR136C	Suppressor of PLc1 deletion	16.35
SPG1	YGR236C	Stationary Phase Gene	11.40
	YBL111C	Helicase-like protein encoded within the telomeric Y' element	6.12
HPF1	YOL155C	Haze Protective Factor	5.88
MPH2	YDL247W	Maltose Permease Homolog	5.72
RGI2	YIL057C	Respiratory growth induced	5.31
PHO89	YBR296C	PHOsphate metabolism	4.35
FMP16	YDR070C	Found in Mitochondrial Proteome	4.19
	YLL066C	Putative Y' element ATP-dependent helicase	3.96
	YPR203W	Putative protein of unknown function	4.03
FRE7	YOL152W	Ferric REductase	3.93
FMP45	YDL222C	Found in Mitochondrial Proteome	3.50
HBT1	YDL223C	Shmoo tip protein	3.46
	YHL050C	Putative protein of unknown function	3.37
YRF1-4	YLR466W	Helicase encoded by the Y' element of subtelomeric regions	3.36
MPH3	YJR160C	Maltose Permease Homolog	3.38
SPG4	YMR107W	Stationary Phase Gene	3.39
YSW1	YBR148W	prospore membrane formation	3.32
	YJL045W	succinate dehydrogenase isozyme	3.34
	YEL077C	Helicase-like protein	3.24
ALD3	YMR169C	ALdehyde Dehydrogenase	3.21
PET117	YER058W	PETite colonies	3.23
FBP1	YLR377C	Fructose-1,6-BisPhosphatase	3.03
	YJL225C	Putative Y' element ATP-dependent helicase	3.00
NRD1	YNL251C	Nuclear pre-mRNA Down-regulation	2.95
RTC3	YHR087W	Restriction of Telomere Capping	2.95
	YNL184C	unknown function	3.00
TKL2	YBR117C	TransKetoLase	2.93
COX17	YLL009C	Cytochrome c OXidase	2.93
CYC1	YJR048W	CYtochrome C	2.89
DIP5	YPL265W	DIcarboxylic amino acid Permease	2.79

RTN2	YDL204W	ReTiculoN-like	2.76
	YEL076C	Putative protein of unknown function	2.78
	YIL177C	Putative Y' element ATP-dependent helicase	2.74
	YBR284W	mutant exhibits longer telomeres	2.68
	YGR067C	Putative protein	2.66
CTT1	YGR088W	CaTalase T	2.66
YRF1-1	YDR545W	Helicase encoded by the Y' element	2.64
SCO1	YBR037C	Suppressor of Cytochrome Oxidase deficiency	2.60
MAM33	YIL070C	Mitochondrial Acidic Matrix protein	2.57
NQM1	YGR043C	Non-Quiescent Mutant	2.51
IFM1	YOL023W	Initiation Factor of Mitochondria	2.51
MRPL19	YNL185C	Mitochondrial Ribosomal Protein, Large subunit	2.50
FET3	YMR058W	FErrous Transport	2.49
TMA10	YLR327C	Translation Machinery Associated	2.46
MDM35	YKL053C-A	Mitochondrial Distribution and Morphology	2.46
SOL4	YGR248W	Suppressor Of Los1-1	2.45
CBP4	YGR174C	Cytochrome B mRNA Processing	2.45
	YHR219W	similarity to helicases	2.44
	YFL066C	Helicase-like protein	2.43
	YCL042W	Putative protein	2.42
	YNL194C	required for sporulation	2.39
SHH4	YLR164W	SDH4 Homolog	2.39
TSL1	YML100W	Trehalose Synthase Long chain	2.35
MRPL40	YPL173W	Mitochondrial Ribosomal Protein, Large subunit	2.34
MRPL49	YJL096W	Mitochondrial Ribosomal Protein, Large subunit	2.33
	YML133C	Putative Y' element ATP-dependent helicase	2.32
MSK1	YNL073W	Mitochondrial aminoacyl-tRNA Synthetase, lysine (K)	2.31
OPT1	YJL212C	OligoPeptide Transporter	2.32
UBP16	YPL072W	UBiquitin-specific Protease	2.30
TFS1	YLR178C	cdc Twenty-Five Suppressor	2.29
GAS2	YLR343W	Glycophospholipid-Anchored Surface protein	2.30
IRC15	YPL017C	Increased Recombination Centers	2.29
DCS2	YOR173W	DeCapping Scavenger	2.27
MRPL31	YKL138C	Mitochondrial Ribosomal Protein, Large subunit	2.26
MEF1	YLR069C	Mitochondrial Elongation Factor	2.25
MRPL6	YHR147C	Mitochondrial Ribosomal Protein, Large subunit	2.25
DON1	YDR273W	DONut	2.26
	YGR053C	Putative protein	2.25
PAI3	YMR174C	Proteinase A Inhibitor	2.24

FYV4	YHR059W	Function required for Yeast Viability	2.23
QRI5	YLR204W	Quasi-Renownless Information	2.22
MRPS9	YBR146W	Mitochondrial Ribosomal Protein, Small subunit	2.21
COX23	YHR116W	Cytochrome OXidase	2.22
HSP82	YPL240C	Heat Shock Protein	2.20
GIP1	YBR045C	Glc7-Interacting Protein	2.20
PMA2	YPL036W	Plasma Membrane ATPase	2.20
CAT2	YML042W	Carnitine AcetylTransferase	2.19
	YMR206W	Putative protein	2.19
HXT5	YHR096C	HeXose Transporter	2.19
CYB2	YML054C	CYtochrome B	2.19
SDH5	YOL071W	Succinate DeHydrogenase	2.17
IDP2	YLR174W	Isocitrate Dehydrogenase, NADP-specific	2.17
	YPR204W	Y' -helicase protein	2.16
AIM17	YHL021C	Altered Inheritance rate of Mitochondria	2.16
NAM9	YNL137C	Nuclear Accommodation of Mitochondria	2.16
PHO5	YBR093C	PHOsphate metabolism	2.16
MZM1	YDR493W	Mitochondrial Zinc Maintenance	2.16
MPC3	YGR243W	Mitochondrial Pyruvate Carrier	2.16
RGI1	YER067W	Respiratory Growth Induced	2.15
MRPL24	YMR193W	Mitochondrial Ribosomal Protein, Large subunit	2.15
EGO4	YNR034W-A	Exit from rapamycin-induced GrOwth arrest	2.13
TIM9	YEL020W-A	Translocase of the Inner Mitochondrial membrane	2.14
MSY1	YPL097W	Mitochondrial aminoacyl-tRNA Synthetase, tyrosine (Y)	2.13
MRPL38	YKL170W	Mitochondrial Ribosomal Protein, Large subunit	2.13
	YLR149C	overexpression causes a cell cycle delay	2.13
PCK1	YKR097W	Phosphoenolpyruvate CarboxyKinase	2.12
HSP42	YDR171W	Heat Shock Protein	2.12
PGM2	YMR105C	PhosphoGlucoMutase	2.11
GDH2	YDL215C	Glutamate DeHydrogenase	2.11
MRPL28	YDR462W	Mitochondrial Ribosomal Protein, Large subunit	2.12
MRPS17	YMR188C	Mitochondrial Ribosomal Protein, Small subunit	2.11
RML2	YEL050C	Ribosomal Mitochondrial Large	2.10
IMD2	YHR216W	IMP Dehydrogenase	2.10
CBP6	YBR120C	Cytochrome B Protein synthesis	2.10
IMG2	YCR071C	Integrity of Mitochondrial Genome	2.10
	YGR021W	unknown function	2.09
TMA17	YDL110C	Translation Machinery Associated	2.09

FMC1	**YIL098C**	**Formation of Mitochondrial Complexes**	**2.08**
RCF1	**YML030W**	**Respiratory superComplex Factor**	**2.07**
TIR4	**YOR009W**	**TIp1-Related**	**2.08**
ARO10	**YDR380W**	**AROmatic amino acid requiring**	**2.07**
SIP4	**YJL089W**	**SNF1-Interacting Protein**	**2.07**
IGD1	**YFR017C**	**Inhibitor of Glycogen Debranching**	**2.06**
ARO9	**YHR137W**	**AROmatic amino acid requiring**	**2.05**
MRP4	**YHL004W**	**Mitochondrial Ribosomal Protein**	**2.05**
MRPL11	**YDL202W**	**Mitochondrial Ribosomal Protein, Large subunit**	**2.05**
RTC6	**YPL183W-A**	**Restriction of Telomere Capping**	**2.06**
LEE1	**YPL054W**	**Zinc-finger protein of unknown**	**2.05**
MRP51	**YPL118W**	**Mitochondrial Ribosomal Protein**	**2.04**
MTO1	**YGL236C**	**Mitochondrial Translation Optimization**	**2.04**
MRPL35	**YDR322W**	**Mitochondrial Ribosomal Protein, Large subunit**	**2.04**
ATG39	**YLR312C**	**AuTophaGy related**	**2.04**
GDB1	**YPR184W**	**Glycogen DeBranching**	**2.03**
MRPS12	**YNR036C**	**Mitochondrial Ribosomal Protein, Small subunit**	**2.04**
	YMR196W	**unknown function**	**2.03**
SRG1	**SRG1**	**SER3 Regulatory Gene**	**2.04**
MST1	**YKL194C**	**Mitochondrial aminoacyl-tRNA Synthetase, Threonine**	**2.03**
SIT1	**YEL065W**	**Siderophore Iron Transport**	**2.03**
MRP1	**YDR347W**	**Mitochondrial Ribosomal Protein**	**2.03**
MRPS5	**YBR251W**	**Mitochondrial Ribosomal Protein, Small subunit**	**2.02**
FMP10	**YER182W**	**Found in Mitochondrial Proteome**	**2.02**
MSS51	**YLR203C**	**Mitochondrial Splicing Suppressor**	**2.02**
ULA1	**YPL003W**	**Ubiquitin-Like protein Activation**	**2.01**
DIN7	**YDR263C**	**DNA Damage INducible**	**2.02**
SSC1	**YJR045C**	**Stress-Seventy subfamily C**	**2.00**
	YBL113C	**Helicase-like protein**	**2.00**
	YDL183C	**KHE system**	**2.01**
	YJL144W	**Cytoplasmic hydrophilin essential**	**2.01**
	YIL055C	unknown function	2.00
CAR1	YPL111W	Catabolism of ARginine	1.99
CMC2	YBL059C-A	Cx9C Motif-Containing protein	1.99
IMA5	YJL216C	IsoMAltase	1.98
MRPL4	YLR439W	Mitochondrial Ribosomal Protein, Large subunit	1.97
ATP12	YJL180C	ATP synthase	1.97
MRPS8	YMR158W	Mitochondrial Ribosomal Protein, Small subunit	1.97
DUR3	YHL016C	Degradation of URea	1.96

PBI1	YPL272C	PSTB2 Interacting protein 1	1.96
PUT4	YOR348C	Proline UTilization	1.95
MRPL37	YBR268W	Mitochondrial Ribosomal Protein, Large subunit	1.95
MRPL13	YKR006C	Mitochondrial Ribosomal Protein, Large subunit	1.94
MRPL25	YGR076C	Mitochondrial Ribosomal Protein, Large subunit	1.94
TCM62	YBR044C	TriCarboxylic acid cycle Mutant	1.93
ECM4	YKR076W	ExtraCellular Mutant	1.93
MRPL1	YDR116C	Mitochondrial Ribosomal Protein, Large subunit	1.93
	YIR014W	unknown function	1.94
RSM27	YGR215W	Ribosomal Small subunit of Mitochondria	1.93
NAM2	YLR382C	Nuclear Accommodation of Mitochondria	1.93
ICL1	YER065C	IsoCitrate Lyase	1.93
MSC1	YML128C	Meiotic Sister-Chromatid recombination	1.92
	YBR285W	unknown function	1.93
RSM28	YDR494W	Ribosomal Small subunit of Mitochondria	1.91
YML6	YML025C	Mitochondrial ribosomal protein	1.91
RSM18	YER050C	Ribosomal Small subunit of Mitochondria	1.91
ADY3	YDL239C	Accumulation of DYads	1.90
LEU1	YGL009C	LEUcine biosynthesis	1.90
MME1	YMR166C	Mitochondrial Magnesium Exporter	1.90
HEF3	YNL014W	Homolog of EF-3	1.90
FLO11	YIR019C	FLOcculation	1.89
MSD1	YPL104W	Mitochondrial aminoacyl-tRNA Synthetase, Aspartate (D)	1.89
MRX14	YDR115W	Mitochondrial oRganization of gene eXpression (MIOREX)	1.89
	YMR090W	unknown function	1.88
RDL2	YOR286W	RhoDanese-Like protein	1.88
RMD9	YGL107C	Required for Meiotic nuclear Division	1.88
ATH1	YPR026W	Acid TreHalase	1.88
UIP4	YPL186C	Ulp1 Interacting Protein	1.87
PFK26	YIL107C	6-PhosphoFructo-2-Kinase	1.87
COX15	YER141W	Cytochrome c OXidase	1.86
PHM7	YOL084W	PHosphate Metabolism	1.86
MSF1	YPR047W	Mitochondrial aminoacyl-tRNA Synthetase, Phenylalanine (F)	1.86
	YMR045C	Retrotransposon TYA Gag and TYB Pol genes	1.85
VMR1	YHL035C	Vacuolar Multidrug Resistance	1.85
IMG1	YCR046C	Integrity of Mitochondrial Genome	1.85
OMS1	YDR316W	OXA1 Multicopy Suppressor	1.85

ATG8	YBL078C	AuTophaGy related	1.85
GRX1	YCL035C	GlutaRedoXin	1.84
MHR1	YDR296W	Mitochondrial Homologous Recombination	1.84
MRPL23	YOR150W	Mitochondrial Ribosomal Protein, Large subunit	1.84
MRP13	YGR084C	Mitochondrial Ribosomal Protein	1.83
GCY1	YOR120W	Galactose-inducible Crystallin-like Yeast protein	1.83
MIA40	YKL195W	Mitochondrial intermembrane space Import and Assembly	1.83
GCV1	YDR019C	GlyCine cleaVage	1.84
ISA2	YPR067W	Iron Sulfur Assembly	1.83
COX11	YPL132W	Cytochrome c OXidase	1.83
ACP1	YKL192C	Acyl Carrier Protein	1.82
AIM18	YHR198C	Altered Inheritance rate of Mitochondria	1.82
ERG28	YER044C	ERGosterol biosynthesis	1.82

Table 6: **WT vs *rtt103*Δ:** List of up-regulated genes in *rtt103*Δ. The cut off employed was >2-fold for up-regulation.

WT vs *rtt103*Δ Down regulated genes

Standard Name	Systematic Name	Description	Fold Change
	YHR218W		0
RTT103	YDR289C	Regulator of Ty1 Transposition	0.01
YRF1-7	YPL283C	Helicase encoded by the Y' element of subtelomeric regions	0.05
URA3	YEL021W	URAcil requiring	0.08
TPO2	YGR138C	Transporter of POlyamines	0.11
HXT4	YHR092C	HeXose Transporter	0.13
MOD5	YOR274W	tRNA MODification	0.14
RDN37-1	RDN37-1	35S ribosomal RNA	0.14
RDN37-2	RDN37-2	35S ribosomal RNA	0.14
MIG2	YGL209W	Multicopy Inhibitor of GAL gene expression	0.15
INO1	YJL153C	INOsitol requiring	0.16
HMS2	YJR147W	High-copy Mep Suppressor	0.21
LOT5	YKL183W	LOw Temperature-responsive	0.21
GCD10	YNL062C	General Control Derepressed	0.22
	YOR338W	Putative protein	0.24
URA10	YMR271C	URAcil requiring	0.25
YPS3	YLR121C	YaPSin	0.27
PTC2	YER089C	Phosphatase Two C	0.29
ARG3	YJL088W	ARGinine requiring	0.30
HUA2	YOR284W		0.30
RFU1	YLR073C	Regulator of Free Ubiquitin chains	0.31
AAH1	YNL141W	Adenine AminoHydrolase	0.31
DML1	YMR211W	Drosophila melanogaster Misato-Like protein	0.32
DBP2	YNL112W	Dead Box Protein	0.33
SEC39	YLR440C	SECretory	0.33
AMD2	YDR242W	AMiDase	0.34
SPO22	YIL073C	SPOrulation	0.34
CEP3	YMR168C	CEntromere Protein	0.36
TOD6	YBL054W	Twin Of Dot6p	0.36
ISM1	YPL040C	Isoleucyl tRNA Synthetase of Mitochondria	0.37
SNR86	snR86		0.37
IMD4	YML056C	IMP Dehydrogenase	0.37
PRS3	YHL011C	PhosphoRibosylpyrophosphate Synthetase	0.37
URA7	YBL039C	URAcil requiring	0.37
RPS21A	YKR057W	Ribosomal Protein of the Small subunit	0.38

ARG1	**YOL058W**	**ARGinine requiring**	**0.39**
NSR1	**YGR159C**		**0.40**
LAS1	**YKR063C**	**Lethal in the Absence of SSD1-v**	**0.40**
	YGR283C		**0.40**
SAM2	**YDR502C**	**S-AdenosylMethionine requiring**	**0.40**
RGT2	**YDL138W**	**Restores Glucose Transport**	**0.40**
SYO1	**YDL063C**	**SYnchronized impOrt or SYmpOrtin**	**0.40**
ITR1	**YDR497C**	**myo-Inositol TRansporter**	**0.41**
	YGR149W	**Putative protein of unknown function**	**0.41**
GRC3	**YLL035W**	**Polynucleotide kinase present on rDNA**	**0.41**
DHR2	**YKL078W**	**DEAH-box RNA helicase**	**0.41**
DBP8	**YHR169W**	**Dead Box Protein**	**0.41**
BNA7	**YDR428C**	**Biosynthesis of NAD**	**0.42**
PRP43	**YGL120C**	**Pre-mRNA Processing**	**0.42**
SDA1	**YGR245C**	**Severe Depolymerization of Actin**	**0.42**
YEA6	**YEL006W**	**Putative mitochondrial NAD+ transporter**	**0.42**
NIP7	**YPL211W**	**Nuclear ImPort**	**0.43**
APT1	**YML022W**	**Adenine PhosphoribosylTransferase**	**0.43**
NVJ3	**YDR179W-A**	**Nucleus-Vacuole Junction**	**0.43**
FAF1	**YIL019W**	**Forty (40) S Assembly Factor**	**0.43**
RPA49	**YNL248C**	**RNA Polymerase A**	**0.43**
	YEL073C	**Putative protein of unknown function**	**0.43**
	YKL069W	**Methionine-R-sulfoxide reductase**	**0.44**
FUI1	**YBL042C**	**5-FlUorourIdine resistance**	**0.44**
DBP3	**YGL078C**	**Dead Box Protein**	**0.44**
TRM82	**YDR165W**	**Transfer RNA Methyltransferase**	**0.44**
FSH1	**YHR049W**	**Family of Serine Hydrolases**	**0.44**
SYC1	**YOR179C**	**Similar to Ysh1 C-terminal**	**0.44**
SSF1	**YHR066W**	**Suppressor of ste4 (Four)**	**0.44**
NSA2	**YER126C**	**Nop Seven Associated**	**0.44**
	YGR079W	**Putative protein of unknown function**	**0.44**
KRR1	**YCL059C**	**contains KRR-R motif**	**0.44**
GPP1	**YIL053W**	**Glycerol-3-Phosphate Phosphatase**	**0.45**
YPQ2	**YDR352W**	**Yeast PQ-loop protein**	**0.45**
TRM5	**YHR070W**	**tRNA Methyltransferase**	**0.45**
KRE29	**YER038C**	**Killer toxin REsistant**	**0.45**
EPT1	**YHR123W**	**EthanolaminePhosphoTransferase**	**0.45**
HTD2	**YHR067W**	**Hydroxyacyl-Thioester Dehydratase**	**0.45**
DIA1	**YMR316W**	**Digs Into Agar**	**0.46**
ELO2	**YCR034W**	**fatty acid ELOngation**	**0.46**

UTP23	**YOR004W**	**U Three-associated Protein**	**0.46**
SRP40	**YKR092C**	**Serine Rich Protein**	**0.46**
	YNL040W		**0.46**
LTV1	**YKL143W**	**Low Temperature Viability**	**0.46**
GEP4	**YHR100C**	**GEnetic interactors of Prohibitins**	**0.46**
SDC1	**YDR469W**	**Set1c, homologue of Dpy30 from C.elegans**	**0.47**
	YPR137C-B		**0.47**
RMT2	**YDR465C**	**aRginine MeThyltransferase**	**0.47**
	YLR036C		**0.47**
HIP1	**YGR191W**	**HIstidine Permease**	**0.47**
HAS1	**YMR290C**	**Helicase Associated with Set1**	**0.47**
NUC1	**YJL208C**	**NUClease**	**0.47**
SYG1	**YIL047C**	**Suppressor of Yeast Gpa1**	**0.47**
	YDR524C-B		**0.47**
ETT1	**YOR051C**	**Enhancer of Translation Termination 1**	**0.47**
PWP1	**YLR196W**	**Periodic tryptophan (W) Protein**	**0.48**
SQT1	**YIR012W**	**Suppressor of QSR1 Truncations**	**0.48**
NUP53	**YMR153W**	**NUclear Pore**	**0.48**
NUG1	**YER006W**	**NUclear GTPase**	**0.48**
UTP11	**YKL099C**	**U Three Protein**	**0.48**
MTR4	**YJL050W**	**Mrna TRansport**	**0.48**
RPA135	**YPR010C**	**RNA Polymerase A**	**0.48**
MKC7	**YDR144C**	**Multicopy suppressor of Kex2 Cold sensitivity**	**0.48**
ADO1	**YJR105W**	**ADenOsine kinase**	**0.48**
EFG1	**YGR271C-A**	**Exit From G1**	**0.48**
UTP8	**YGR128C**	**U Three Protein**	**0.49**
SMP3	**YOR149C**	**Stable Maintenance of pSRI**	**0.49**
CNS1	**YBR155W**	**CyclophiliN Seven suppressor**	**0.49**
PIR3	**YKL163W**	**Protein containing Internal Repeats**	**0.49**
KRE33	**YNL132W**	**Killer toxin REsistant**	**0.49**
OPI3	**YJR073C**	**OverProducer of Inositol**	**0.49**
ASC1	**YMR116C**	**Absence of growth Suppressor of Cyp1**	**0.49**
BMT5	**YIL096C**	**Base Methyltransferase of Twenty five S rRNA 5**	**0.49**
	YHR033W		**0.49**
PTI1	**YGR156W**	**PTa1p Interacting protein**	**0.49**
RGS2	**YOR107W**	**Regulator of heterotrimeric G protein Signaling**	**0.49**
RIM13	**YMR154C**	**Regulator of IME2**	**0.49**
RAS1	**YOR101W**	**homologous to RAS proto-oncogene**	**0.49**
SFG1	**YOR315W**	**SuperFicial pseudohyphal Growth**	**0.49**
FMP41	**YNL168C**	**Found in Mitochondrial Proteome**	**0.49**

DPM1	**YPR183W**	**Dolichol Phosphate Mannose synthase**	**0.49**
ENP2	**YGR145W**	**Essential Nuclear Protein**	**0.50**
RPL7B	**YPL198W**	**Ribosomal Protein of the Large subunit**	**0.50**
MIS1	**YBR084W**	**MItochondrial C1-tetrahydrofolate Synthase**	**0.50**
DUS3	**YLR401C**	**DihydroUridine Synthase**	**0.50**
NOP56	**YLR197W**	**NucleOlar Protein of 56.8 kDa**	**0.50**
DPH1	**YIL103W**	**DiPHthamide biosynthesis**	**0.50**
BFR2	**YDR299W**	**BreFeldin A Resistance**	**0.50**
POL5	**YEL055C**	**POLymerase**	**0.50**
FMS1	**YMR020W**	**Fenpropimorph-resistance Multicopy Suppressor**	**0.50**
AAC1	**YMR056C**	**ADP/ATP Carrier**	**0.50**
RPS27A	**YKL156W**	**Ribosomal Protein of the Small subunit**	**0.50**
NOP1	**YDL014W**	**NucleOlar Protein**	**0.50**
	YDR415C		**0.50**
NOP14	**YDL148C**	**NucleOlar Protein**	**0.50**
SAM3	**YPL274W**	**S-AdenosylMethionine metabolism**	**0.50**
NOG2	**YNR053C**	**NucleOlar G-protein**	**0.50**
	YMR310C		**0.50**
TPA1	YER049W	Termination and PolyAdenylation	0.51
HFM1	YGL251C	Helicase Family Member	0.51
RNT1	YMR239C	RNase Three	0.51
UTP9	YHR196W	U Three Protein	0.51
ROK1	YGL171W	Rescuer Of Kem1	0.51
	YPL109C		0.51
PPX1	YHR201C		0.51
BUD23	YCR047C	BUD site selection	0.51
NOP13	YNL175C	NucleOlar Protein	0.52
TRM11	YOL124C	TRna Methyltransferase	0.52
CPA1	YOR303W	Carbamyl Phosphate synthetase A	0.52
TCD2	YKL027W	tRNA ThreonylCarbamoyladenosine Dehydratase	0.52
BCP1	YDR361C		0.52
UTR2	YEL040W	Unidentified TRanscript	0.52
	YLR287C		0.52
FET4	YMR319C	FErrous Transport	0.52
NOG1	YPL093W	NucleOlar G-protein	0.52
UTP18	YJL069C	U Three Protein	0.52
NSA1	YGL111W	Nop Seven Associated	0.52
YTM1	YOR272W		0.52
INO2	YDR123C	INOsitol requiring	0.52

ARX1	YDR101C	Associated with Ribosomal eXport complex	0.52
RPL8A	YHL033C	Ribosomal Protein of the Large subunit	0.53
IPI1	YHR085W	Involved in Processing ITS2	0.53
RPC40	YPR110C	RNA Polymerase C	0.53
RPC34	YNR003C	RNA Polymerase C	0.53
ATG5	YPL149W	AuTophaGy related	0.53
	YGL117W		0.53
PUS1	YPL212C	PseudoUridine Synthase	0.53
NOP15	YNL110C	NucleOlar Protein	0.53
CIC1	YHR052W	Core Interacting Component	0.53
SOK2	YMR016C	Suppressor Of Kinase	0.53
CPT1	YNL130C	CholinePhosphoTransferase	0.53
TPO4	YOR273C	Transporter of POlyamines	0.53
EBP2	YKL172W	EBNA1-binding protein (homolog)	0.53
EMG1	YLR186W	Essential for Mitotic Growth	0.53
FOL1	YNL256W	FOLic acid synthesis	0.53
RPA34	YJL148W	RNA Polymerase A	0.53
RPL16A	YIL133C	Ribosomal Protein of the Large subunit	0.53
RRB1	YMR131C	Regulator of Ribosome Biogenesis	0.54
ELO3	YLR372W	fatty acid ELOngation	0.54
RPL5	YPL131W	Ribosomal Protein of the Large subunit	0.54
EFM1	YHL039W	Elongation Factor Methyltransferase	0.54
NOP7	YGR103W	NucleOlar Protein	0.54
UTP4	YDR324C	U Three Protein	0.54
ARG5,6	YER069W	ARGinine requiring	0.54
GUA1	YMR217W	GUanine Auxotroph	0.54
TSR2	YLR435W	Twenty S rRNA accumulation	0.54
ESF1	YDR365C	Eighteen S rRNA Factor	0.54
KRS1	YDR037W	Lysyl (K) tRNA Synthetase	0.54
RRP5	YMR229C	Ribosomal RNA Processing	0.54
PHR1	YOR386W	PHotoreactivation Repair deficient	0.54
NOP8	YOL144W	NucleOlar Protein	0.55
SNT2	YGL131C		0.55
TTI1	YKL033W	Two Tel2-Interacting protein	0.55
PGA3	YML125C	Processing of Gas1p and ALP	0.55
ARG4	YHR018C	ARGinine requiring	0.55
RIX7	YLL034C	RIbosome eXport	0.55
NRP1	YDL167C	N (asparagine)-Rich Protein	0.55
RRP12	YPL012W	Ribosomal RNA Processing	0.55
	YBL055C		0.55

ERB1	YMR049C	Eukaryotic Ribosome Biogenesis	0.55
TRM10	YOL093W	Transfer RNA Methyltransferase	0.55
HPM1	YIL110W	Histidine Protein Methyltransferase	0.55
SER3	YER081W	SERine requiring	0.55
DRS1	YLL008W	Deficiency of Ribosomal Subunits	0.55
GWT1	YJL091C	GPI-anchored Wall protein Transfer	0.55
NUP82	YJL061W	NUclear Pore	0.56

Table 7: **WT vs *rtt103*Δ:** List of down-regulated genes in *rtt103*Δ. The cut-off employed was <0.5 (2-fold) for down-regulation.

CHAPTER 5:

Loss of Rtt103 leads to reduced global translation

5.1 Introduction

The growth and survival of unicellular organisms depends on their ability to adapt to varying environments in their ecological niche, including temperature downshifts. Low temperature can affect multiple biochemical and physiological properties of a cell, including membrane rigidification, dysregulation of protein folding, topological changes in DNA, stabilization of secondary structures in RNA, and poor translational efficiency[173,174]. Besides, it is thermodynamically unfavorable for many protein-protein interactions, thus adversely affecting the assembly of large multi-subunit complexes. The large macromolecular ribonucleoprotein complexes of splicing and ribosome machinery are those that are directly affected by the magnitude of temperature downshift[175–178]. In bacteria, a significant proportion of cold-sensitive mutants were found to contain mutations that affect ribosome synthesis, making them unable to grow on complex media at reduced temperatures[177,179]. Initially, cold sensitivity was used as a phenotype to isolate mutants defective in ribosome biogenesis[176–178]. In vitro reconstitution studies regarding 30S ribosomal particles assembly have suggested that indeed it is a temperature-dependent process with an Arrhenius activation energy of 38kcal/mole. At reduced temperatures, i.e. at 10°C or below, this process was infinitely slow and accumulated intermediate 21S particles[180]. This is further confirmed by the fact that many of the ribosomal assembly mutants were observed to be cold-sensitive but viable at higher temperatures[176].

Ribosome biogenesis is an energy-intensive process requiring a highly coordinated cascade of events. In an actively dividing cell, 60% of the total transcription machinery is devoted to rRNA synthesis and approximately 2000 ribosomes are produced per minute[168]. A functional ribosomal maturation involves four ribosomal RNA (rRNA) and 80 ribosomal proteins (RPs) aided by more than 200 trans-acting assembly factors (Afs) at various stages of pre-ribosomal maturation[181,182]. Although the association of Afs is transient and occurs across

three cellular compartments, the exact molecular mechanism and spatiotemporal regulation of this event still remains elusive.

The economics of ribosome biogenesis determines the active growth potential of a cell; hence, it is tightly regulated and well-coordinated at each stage. The first step involves the transcription of ribosomal RNA (rRNA) by RNA Pol I (35S) and RNA Pol III (5S). Second, the nascent 35S pre-rRNA is cleaved into early precursors for small subunit (18S rRNA) and large subunit (5.8S and 25S rRNA) by the coordinated action of various exo- and endo-nucleases[183]. The third step involves the chemical and secondary modifications of rRNA, including methylation and pseudouridylation guided by various small nucleolar RNAs (snoRNAs), methyltransferases, and other modification enzymes[184,185]. Apart from the ones described above, a vast number of energy-consuming proteins, such as RNA helicase, GTPases, AAA-ATPases, and dedicated chaperones, contribute to the assembly[186–188]. The final step involves the association of export adapters with the pre-ribosomal particles in the nucleus, ensuring their active transport into the cytoplasm, where the final maturation and quality control take place[189,190].

From the existing literature, it is now evident that mutations in any of the critical players described above can result in cold sensitivity due to defects in the ribosomal assembly. For example, dominant mutations in rRNA, which affect the intramolecular base pairing or increase the stability of mispaired RNA intermediates, result in improper rRNA folding, ultimately leading to cold sensitivity[191]. Defects in pre-rRNA processing as in yeast *nsr1* mutants, the structural homolog of mammalian nucleolin, also display cold-sensitivity due to accumulation of unprocessed 20S[191]. Apart from these, there are several reports on deletion of single ribosomal encoding proteins or single amino acid change in these proteins that confer cold sensitivity across a wide range of organisms[192–196]. Over the span of 50 years, most of our current understanding of the significant events involved in ribosome biogenesis have stemmed majorly from studies carried out using yeast and bacteria. Attempts to isolate suppressors of the defective ribosome biogenesis-induced cold sensitivity have paved the way for identifying many key regulators in ribosome biogenesis[197,198]. Recent mounting evidence suggest that ribosomes are not monolithic machines; instead, these are a heterogeneous populations, which have a differential stress and tissue-specific translational preference, making it further difficult to decipher this pathway in detail[199–201].

In *Saccharomyces cerevisiae*, so far, 323 genes have been reported to be cold-sensitive (Saccharomyces Genome Database). This accounts for roughly 5% of the 6485 annotated genes in the genome. Our functional clustering of the cold-sensitive genes revealed a significant correlation between cold sensitivity and ribosome biogenesis (Figure 5.1). In this study, we report that three more mutants, namely *rtt103*Δ, *rai1*Δ, and *rat1-1*, are cold-sensitive and we further show that this is due to defects in ribosome biogenesis and reduced global translation. We have explored the novel role of transcription termination complex Rtt103-Rai1-Rat1 in ribosome biogenesis via isolation of extragenic suppressors.

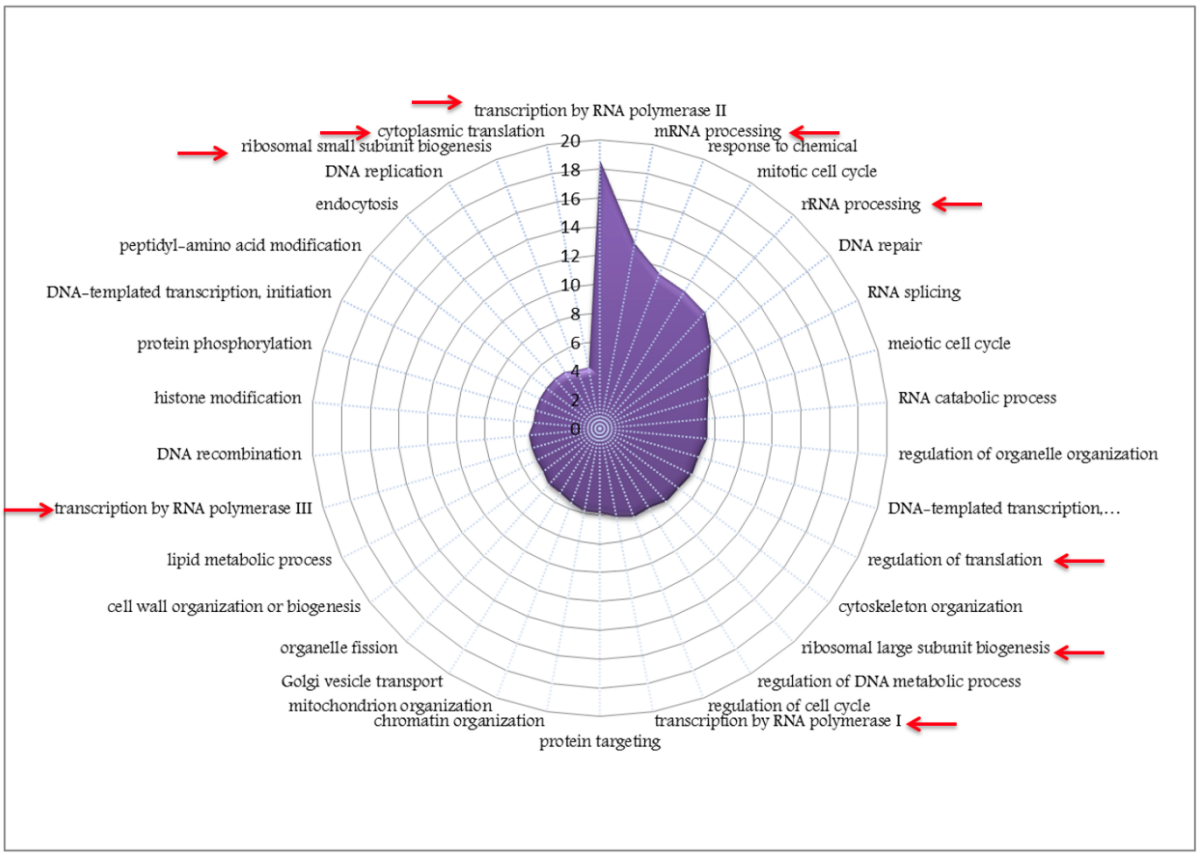

Figure 5.1: **Functional categorisation of cold-sensitive genes in yeast:** In *Saccharomyces cerevisiae,* 323/6485 annotated genes are known to be cold-sensitive. Functional categorisation has revealed that the majority of these genes are involved in the regulation of ribosome biogenesis (highlighted with red arrows).

5.2 Results

*rtt103*Δ, *rai1*Δ, and *rat1-1* are cold sensitive

Previous investigations from our laboratory have reported that overexpression of Rtt103 can partially suppress the temperature and MMS sensitivity of *yku70*Δ[81]. Hence, we extended our study to assess whether Rtt103 plays any role in telomere metabolism or NHEJ-like Yku70. As described in Chapter 3, we have uncovered a novel role of the transcription termination complex Rtt103-Rai1-Rat1 in sub-telomeric gene silencing and co-transcriptional regulation of long-non coding RNA's (lncRNA's) arising from the sub-telomeric and telomeric tracts[202]. In order to gain molecular insights into the DNA repair mechanism, our preliminary assessment with *rtt103*Δ *yku70*Δ double mutant displayed an exacerbated temperature sensitivity and DNA damage sensitivity phenotype implying the fact that they might function in two independent pathways to maintain genome integrity[81]. Hence, we tested whether overexpression of *RTT103* can suppress any of the other phenotypes exhibited by NHEJ mutants (data not shown). Although we did not find suppression of any of the other phenotypes (MMS, UV, or Temperature), we intriguingly found that *rtt103*Δ mutants were cold sensitive (16°C). Furthermore, the cold sensitivity was also seen in the mutants of its interacting partners, namely, *rai1*Δ and *rat1-1*. Overexpression of *RTT103*, either under its own promoter (2μ) YEP-*RTT103* or from a strong constitutive TEF1 promoter (pBevyT-*RTT103*) rescued the cold sensitivity exhibited by *rtt103*Δ mutants. However, overexpression of *RTT103* did not alter the cold sensitivity of either *rai1*Δ or *rat1-1* (Figure 5.2A and B).

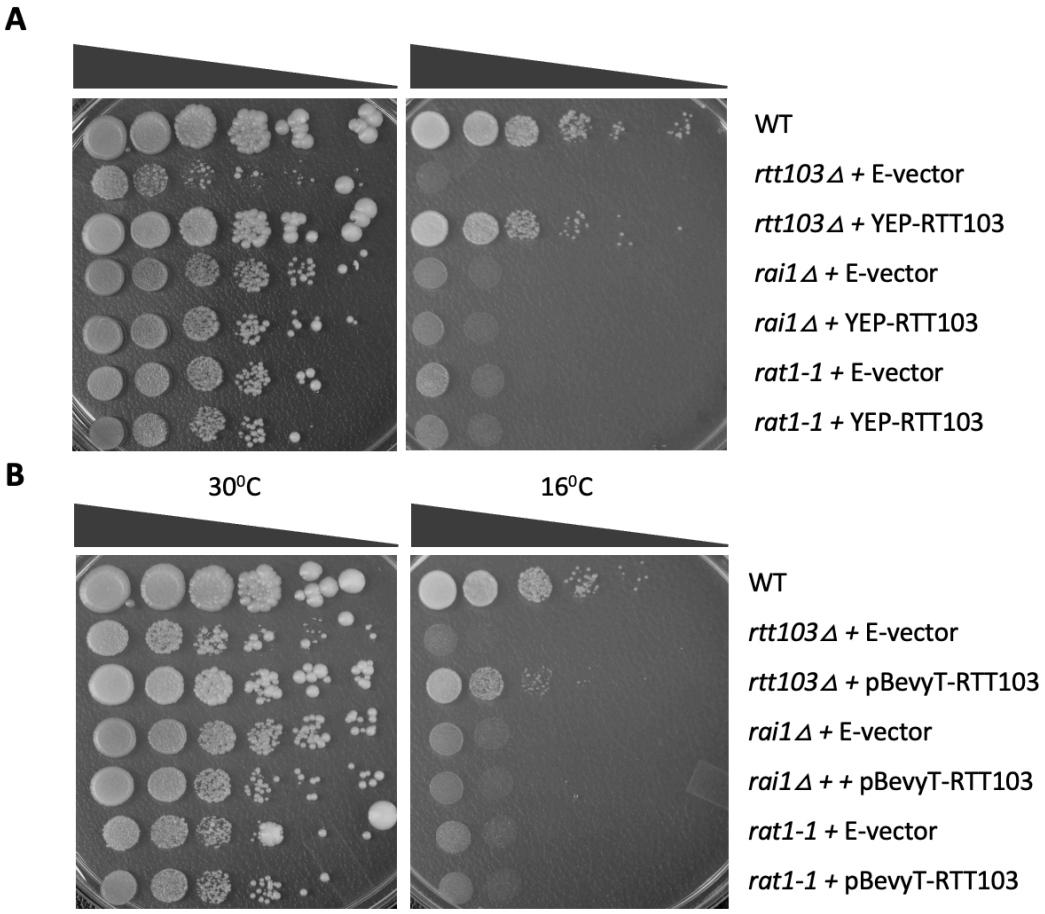

Figure 5.2: ***rtt103*Δ, *rai1*Δ, and *rat1-1* are cold sensitive:** A and B. WT, *rtt103*Δ, *rai1*Δ, and *rat1-1* strains harbouring either (2μ) YEP-*RTT103*, pBevyT-*RTT103*, or empty vector (E-vector) were grown overnight in SC-TRP broth and 5 μl of 10-fold serial dilution was spotted on SC-TRP. The plates were imaged after five days of incubation at 30°C and 16°C.

Ribosome biogenesis is down-regulated in *rtt103*Δ

Data from a study that profiled gene expression changes over time when yeast were exposed to low temperatures showed that ~25% of the genome had altered expression under reduced temperature[203]. Time-dependent analysis revealed that molecular adaptation happens in three different phases. In the early phase, all the genes related to rRNA processing and RNA Pol I subunits were up-regulated >1.5 fold. In the middle phase, the majority of the upregulated genes were cytosolic ribosomal protein (RPs) genes. Based on the expression profile, it was evident that all the 94 up-regulated genes (40 small subunit RPs / 54 large subunit RPs) were

cooperatively regulated >2 fold. In the late phase, genes related to signal transduction, stress-responsive elements (STREs), metabolism, and cell rescue were upregulated, indicating that prolonged stress might trigger the expression of genes responsible for survival in lower temperatures. Accounting for the fact that reduction in temperature results in poor translation, the coordinated upregulation of RPs only during the middle phase might serve as an adaptive strategy to upregulate STREs for late-phase adaptation specifically[32].

From our RNA-seq analysis, we have found that 60% of the genes downregulated in *rtt103*Δ could be classified under the category of rRNA processing (Figure 4.6A and B). As upregulation of ribosome biogenesis is a key first response to shifting yeast cells to low temperatures, we wondered if *rtt103*Δ, with an already compromised expression of ribosomal biogenesis genes, would be particularly susceptible to cold temperatures. To test this and also have a holistic view of the translational status, *rtt103*Δ and *rai1*Δ were subjected to polysome analysis with and without transient exposure (2 hours) to 16°C. Logarithmically grown WT, *rtt103*Δ, and *rai1*Δ cells ($O.D_{600}$ 0.8-1.0) were treated with cycloheximide (100 μg/ml) before harvesting and subjected to lysis followed by sucrose density gradient centrifugation. The distribution of free ribosomal subunits, monosomes, and polysomes was determined by the absorbance value obtained from the fractionated profiles. In comparison to the wild type, both *rtt103*Δ and *rai1*Δ, exhibited an altered ratio of 60S to 40S subunits. Even under unperturbed conditions (30°C), both the mutants revealed a higher fraction of monosomes indicating a global translational repression, which is consistent with the reduction in polysome peaks (Figure 5.3 A-C). The reduction in polysome fraction was more pronounced in case of *rai1*Δ compared to *rtt103*Δ, suggesting that the translational block was more severe in *rai1*Δ. This effect was further exacerbated at reduced temperature (16°C). Although the monosome fraction was lowered upon cold shock in both mutants, it did not significantly increase the active polysome fraction (Figure 5.3 D-F). This is possibly because of lowered translation initiation in the mutants compared to wild type cells.

Overall, the above results indicate that the translational capacity is compromised in *rtt103*Δ and *rai1*Δ and 40S subunit accumulation is further exacerbated upon reduced temperature. This suggests that the translation initiation and /or assembly of ribosomal subunits is compromised in these mutants at 16°C.

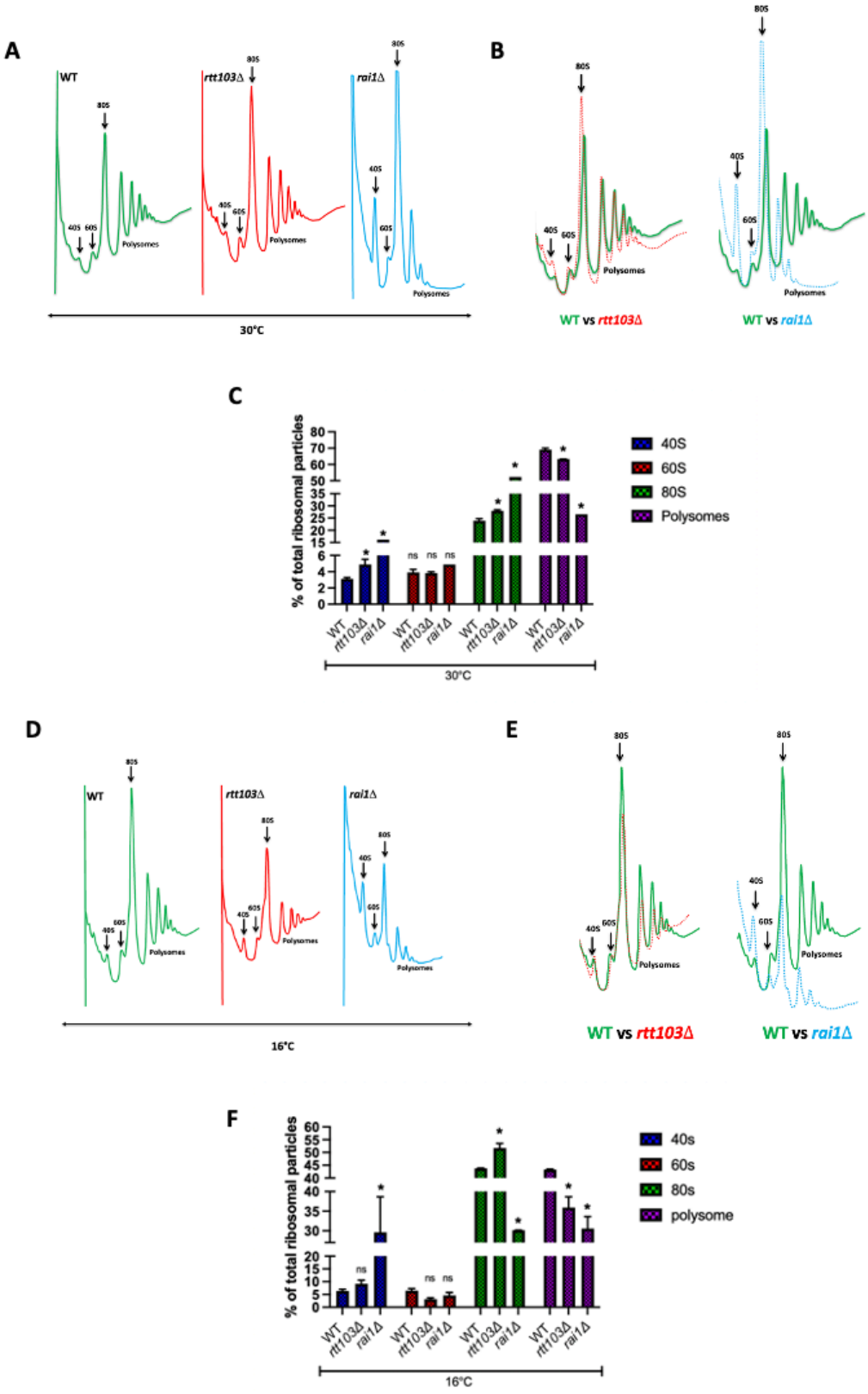

Figure 5.3: ***rtt103*Δ and *rai1*Δ display translational attenuation:** A and D- Polysome profile analysis of WT, *rtt103*Δ, and *rai1*Δ at 30°C or after a transient shift to 16°C for 2 hours. The optical density profiles (OD_{A254}) of the polysome gradient fraction are represented with arrows

indicating individual peaks corresponding to 40S, 60S, 80S (monosomes), and polysomes. B and E- Overlap of the absorption profiles of *rtt103*Δ and *rai1*Δ in comparison to wild type. C and F- Quantification of individual peaks calculated as the total area and mean value from two experiments is represented.

Restoring rRNA biogenesis via RNA Pol II driven promoter suppresses the cold sensitivity of *rtt103*Δ and *rai1*Δ

In *Saccharomyces cerevisiae*, the ribosomal rRNA is encoded by the *RDN1* locus and synthesized as two separate transcripts, namely 35S and 5S, by RNA Pol I and Pol III, respectively. The 35S pre-rRNA is further processed into 18S, 5.8S, and 25S by a series of transcriptional and co-transcriptional events[183]. The *RDN1* locus represents 10% of the entire genome (1-2Mb) arranged as a single tandem array of ~100-150 copies on chromosome XII. In an actively dividing cell, 60% of the total transcription is carried out by RNA Pol I, which is devoted to rRNA[168]. In an actively dividing cell, the relative distribution of RNA species is 80% rRNA, 15% tRNA, and 5% mRNA.

From our RNA Seq data, it was quite evident that both significant players of rRNA transcription – RNA Pol I (4) and RNA Pol III (3) subunits- were substantially downregulated in *rtt103*Δ (Figure 4.8 and Table 4.10). Hence, the drastic reduction of rRNA levels (shown in Figure 4.8B) might be a direct consequence of reduced transcription of rDNA. Although several other vital players in ribosome biogenesis were downregulated in *rtt103*Δ, including RPs, chaperones, and nucleolar proteins (NOPs), owing to the fact that rRNA biogenesis and transcription of RPs are tightly interlinked, we attempted to increase the rRNA biogenesis[204,205]. We ectopically overexpressed 35S rRNA under the control of RNA Pol II driven galactose inducible promoter. *rtt103*Δ and *rai1*Δ harbouring pNOY353 (GAL7-35S rDNA, 5S rDNA) or empty vector were grown overnight on SC-TRP complete medium and spotted on SC-TRP raffinose or SC-TRP galactose. The raffinose medium would serve as neither a repressible nor an inducible carbon source, thereby resulting in a low level of induction. Whereas, in the presence of galactose, a strong induction of GAL7-35S rDNA is observed. As seen in Figure 5.4, increased expression of rRNA suppressed the cold sensitivity exhibited by both *rtt103*Δ and *rai1*Δ. The suppression was more evident on the raffinose plate than on the galactose plate

at 16°C. This further confirms the notion that cells have evolved mechanisms to maintain equimolar amounts of RPs to rRNA. Extensive overexpression of rRNA under galactose might have titrated away the already less available RPs in *rtt103*Δ and *rai1*Δ resulting in toxicity, whereas the wild type remained unaffected.

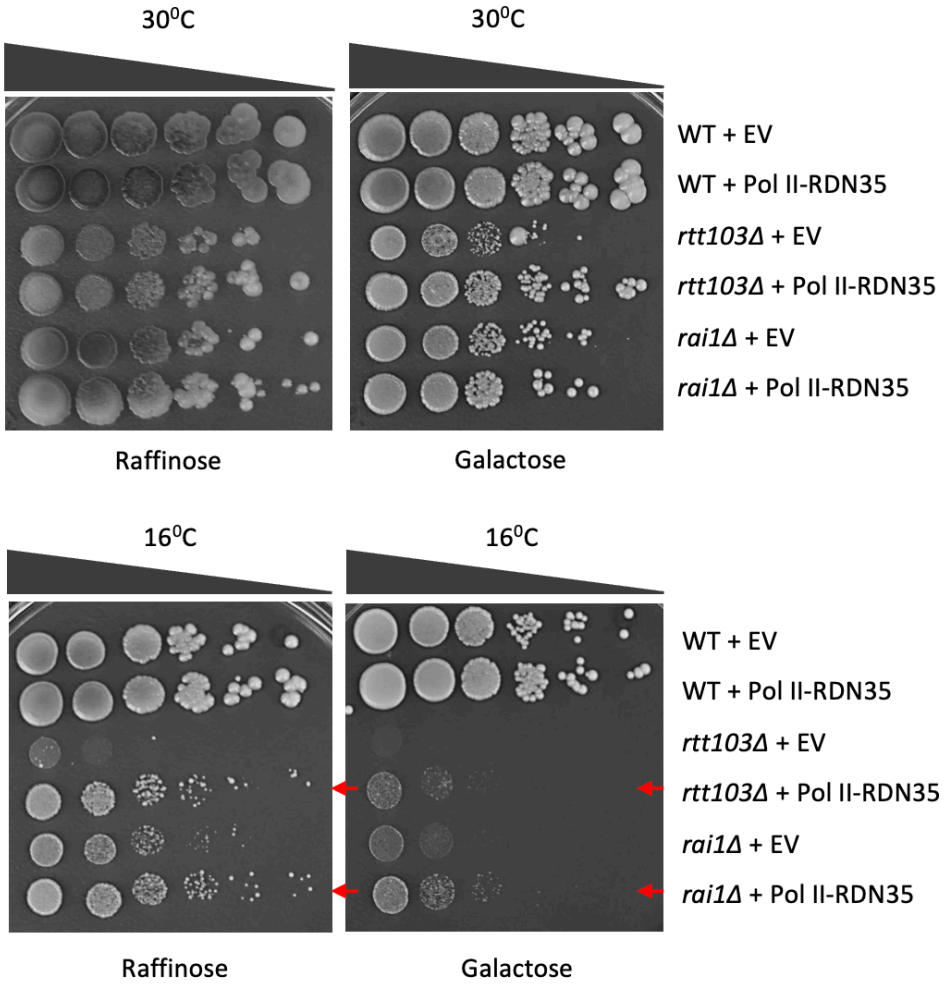

Figure 5.4: **Expression of rRNA via RNA Pol II-driven promoter reduces the cold sensitivity of *rtt103*Δ and *rai1*Δ:** WT, *rtt103*Δ, and *rai1*Δ strains were transformed with either empty vector or plasmids carrying rDNA under the control of RNA Pol II driven galactose inducible promoter were grown overnight in SC-TRP broth and 5 µl of 10-fold serial dilution was spotted on SC-TRP Raffinose and SC-TRP Galactose. The plates were imaged after five days of incubation at 30°C and 16°C.

Our results so far indicate that the growth defect exhibited by *rtt103*Δ and *rai1*Δ at reduced temperature is due to impaired translation. Both the mutants display dysregulation of several RNA Pol I (4) and RNA Pol III (3) subunits and their subsequent reduction in rRNA synthesis. The overexpression of rRNA under the control of RNA Pol II rescues cold sensitivity. In support of this notion, we assessed the sensitivity of these mutants to translational inhibitor hygromycin, which inhibits peptidyl translocation in translation[206]. Mutant of RNA Pol I subunit, Rpa49 and Nsr1, which are involved in pre-rRNA processing, were used as a positive control; this was also found to be downregulated in *rtt103*Δ from our RNA Seq data and *rdn*Δ+ pRDN-hyg1 as the negative control. As seen in Figure 5.4, *rtt103*Δ, *rai1*Δ, and *rat1-1* displayed varying levels of sensitivity to increasing concentrations of hygromycin. The sensitivity was in direct correlation with the levels of defect in ribosome biogenesis, as seen in our polysome analysis (Figure 5.3). *rai1*Δ exhibited more sensitivity even at lower concentrations than *rtt103*Δ.

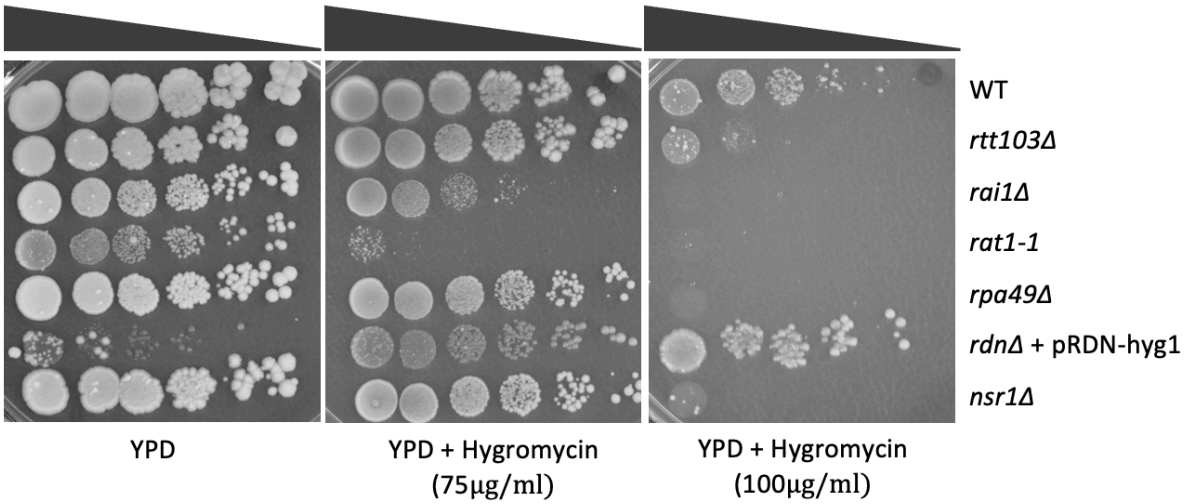

Figure 5.5: ***rtt103*Δ, *rai1*Δ, *and rat1-1* are sensitive to translational inhibitors:** The indicated strains were grown overnight in YPD broth, and 5 µl of 10-fold serial dilution was spotted on YPD and YPD + Hygromycin (75/100 µg/ml). The plates were imaged after three days of incubation at 30°C.

Multi-copy suppressor screen for *rtt103*Δ cold sensitivity

From our preliminary characterization, it was evident that the cold sensitivity exhibited by *rtt103*Δ and *rai1*Δ was due to reduced rRNA synthesis and dysregulation of ribosome biogenesis. Although we have succeeded in partially suppressing the cold sensitivity via overexpressing rRNA, we screened for more extragenic suppressors via genomic library overexpression. This might provide molecular insights into other active players that participate in regulating ribosome biogenesis at reduced temperatures. *rtt103*Δ was transformed with genomic library cloned in 2μ vector YEplac113 (multicopy suppressor) and scored for early emergers at 16°C. A total of 88 early emergers were selected for further screening. A screening PCR was set up to eliminate *RTT103* from the suppressor pool, and we found 86/88 were *RTT103*. In some of the suppressors, just 882bp/1230bp of *RTT103* was able to suppress the cold sensitivity, indicating that C-terminal domain of *RTT103* is dispensable for suppression.

BFR1 and TSR4 are potential multi-copy suppressors for *rtt103*Δ cold sensitivity

The remaining two suppressors possessed large fragments of genomic DNA encompassing multiple genes in between them, as summarized in Table 5.4. After sub-cloning, the suppressors were found to be *BFR1* and *TSR4*, respectively. Although they were obtained as the suppressors for *rtt103*Δ cold sensitivity, we extended our study to test whether they could suppress *rai1*Δ and *rat1-1*. Both *BFR1* and *TSR4* suppressed *rtt103*Δ and *rai1*Δ cold sensitivity, but not *rat1-1*. This implies that Rat1 exonuclease activity might be crucial for surviving at reduced temperatures. From previous literature, it was quite evident that Rat1 plays critical roles in ribosome biogenesis, including rRNA maturation and splicing of introns from RPs[63,207,208].

S. No.	Genome Coordinates and Size	Enclosed Genes	Actual Suppressor
1	717930-723742 Chr XV (5.81kb)	~~MCA1 (1299bp)~~ BFR1 YOR199W YOR200W MRM1 HIS3 YOR203W ~~DED1 (1815bp)~~	BFR1
2	277164-283487 Chr XV (6.33kb)	~~YOLO24W (519bp)~~ ARS1511 IFM1 TSR4 SUF17 ~~DIS3 (3006bp)~~	TSR4

Table 7: **List of suppressors obtained in this study:** This table indicates the details of suppressors obtained for *rtt103*Δ cold sensitivity.

BFR1 and the mechanism of suppression

BFR1 was initially identified in a genetic screen as a multi-copy suppressor of brefeldin A-induced lethality in yeast[209]. Later, it was found to be associated with Scp160 in mRNP-assisted polyribosome complexes[210,211]. This association is RNA dependent, and the absence of *bfr1* results in the loss of Scp160 association with the mRNP complex[210]. Although Bfr1 lacks the classical RNA binding domain, several experimental evidences have confirmed its

association with a subset of mRNA's[212–214]. Under normal conditions, Scp160 and Bfr1 work together by preventing the access of polysome-associated mRNP complexes to P-bodies, whereas under prolonged stress conditions, Bfr1 targets translationally repressed mRNA into P-bodies and regulates their turnover[205]. Because of this dynamic behaviour, it also localizes to the cytoplasm besides its predominant ER localization. Loss of either Bfr1 or Scp160 displays similar phenotypes like altered cell morphology, change in ploidy, induction of P-bodies, and impaired nuclear segregation. Taken altogether, this suggests a role for Bfr1 in regulating mRNA turnover via P-bodies[210]. Hence, we assessed the status of P-bodies in *rtt103*Δ and *rai1*Δ.

Status of P-body formation in *rtt103*Δ and *rai1*Δ

Processing bodies (P-bodies) are membrane-less biomolecular condensates, which play a significant role in the regulation of eukaryotic gene expression. These are highly dynamic structures induced by a wide variety of stresses and vary in number and size[86,215]. The major constituents of P-bodies include proteins involved in translational repression and mRNA decay machinery[216]. In higher eukaryotes, the mRNA decay machinery is majorly dependent on two pathways, viz. P-bodies and exosomes. In the P-body, the mRNAs are degraded in bulk from the 5′ to 3′ direction, whereas in the exosome, the degradation occurs in the 3′ to 5′ direction.[5,217] Bfr1 is known to relocalize translationally repressed mRNAs to P-bodies[218]. Our polysome profiling data indicate that there is translational repression in *rtt103*Δ and *rai1*Δ. Therefore, we reasoned that there may be altered levels of P bodies in these mutants. To examine P bodies, we employed Edc3-mCherry as a marker, which functions as an enhancer for mRNA decapping machinery and is localized onto the P-bodies. First, we examined the logarithmically grown cells of WT, *rtt103*Δ, and *rai1*Δ (O.D-0.8-1.0) harbouring Edc3-mCherry via fluorescence microscopy. As seen in Figure 5.6A, a significant population of both *rtt103*Δ and *rai1*Δ displayed increased P-body formation even under unperturbed conditions (30°C). This is consistent with the observation that overall translational status is compromised in both *rtt103*Δ and *rai1*Δ and this stress could lead to the accumulation of such translationally repressed mRNA to the P -bodies, thereby increasing the number of P-bodies. Prolonged exposure to reduced temperature further exacerbated the P-body size and number, as shown in Figure 5.6A and B.

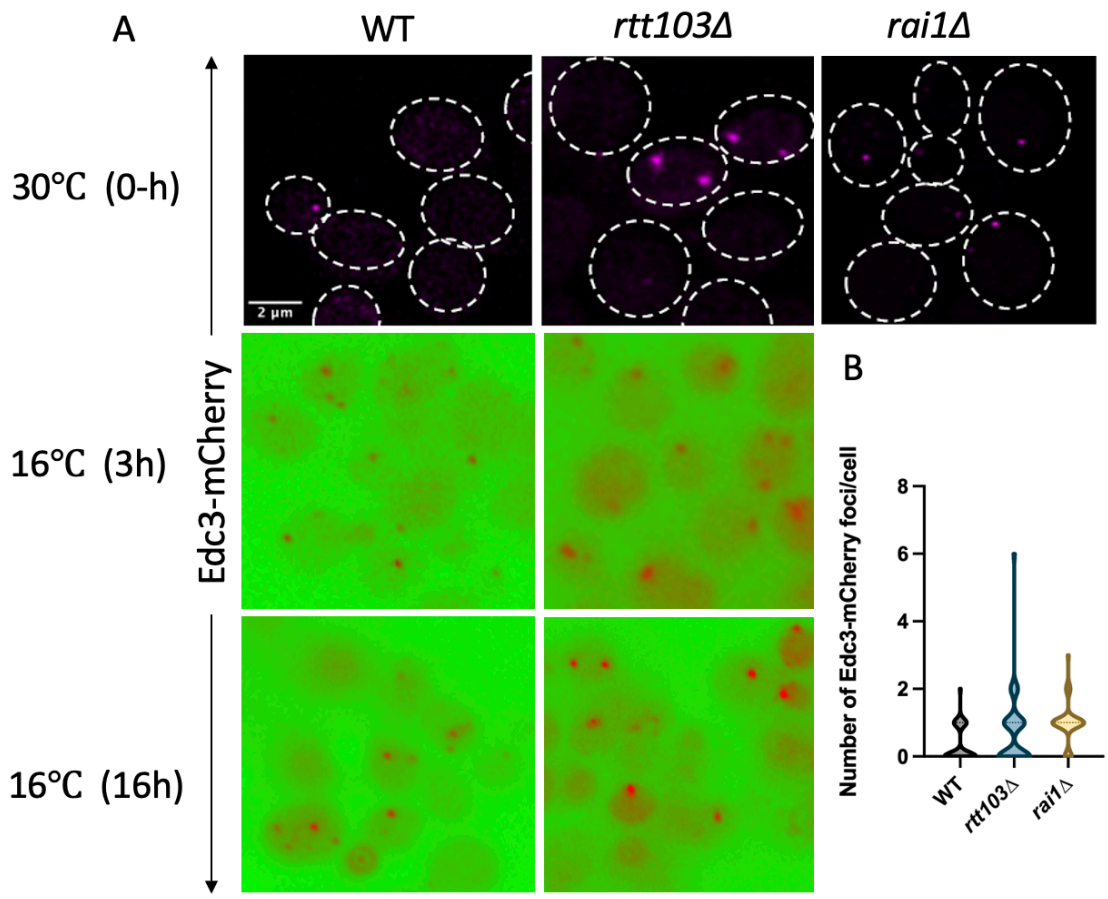

Figure 5.6: **Status of P-Body formation in *rtt103*Δ and *rai1*Δ:** A. Distribution of Edc3-mCherry foci in *rtt103*Δ *and rai1*Δ under unperturbed conditions and upon exposure to reduced temperature. The images are depth colour-coded to represent the P-body accumulation over time. All panels are of the same magnification; scale bar equals to 2 µm and is shown in the top left panel. B. Violin plot representing the number of P-Bodies per cell (n=100).

BFR1 alters the dynamics of P-body formation and improves the active translation in both *rtt103*Δ and *rai1*Δ

P-bodies are known to associate with a range of RNA-binding proteins, which suggests many mRNAs are localized here, and further implies that this localization might be regulated differently. For instance, Bfr1 relocates late-phase mRNAs into P-bodies upon prolonged glucose starvation[218]. According to the existing literature, the number and size of P-bodies are directly correlated with the type of stress. For example, the number of P-bodies increase dramatically under conditions that inhibit translation initiation, such as glucose deprivation and osmotic stress. Conversely, heat or oxidative stress, which does not affect translation rate, does not result in an increase in the number of P-bodies[215]. Having established that there is an increased number of P-bodies, possibly due to translational repression in *rtt103*Δ and *rai1*Δ, we speculated that there may be potentially two mechanisms underlying the suppression of cold sensitivity by Bfr1. First, it might be the effective sequestration of translationally repressed mRNAs into P-bodies. Effective sequestration of the pre-stress pool of mRNAs might provide a selective advantage for the cell to reprogram the translation of specific mRNAs, reflecting the new growth condition. For instance, it is well established that P-bodies are not mere sites for mRNA decay and the sequestered mRNAs can re-enter the active translational pool once the stress is alleviated[217,219].

Secondly, P-bodies do not contain ribosomal proteins and further aggregation cannot occur until the P-body mRNPs are free of ribosomal proteins[215,217,220]. Treatment of yeast cells with cycloheximide - a potent inhibitor of translational elongation, which results in mRNA accumulation on polysomes - results in defective P-body formation[215]. We wondered if the effective sequestration of translationally repressed mRNAs by Bfr1 into P-bodies might free the stalled ribosomes, which can now be utilized for translating stress-specific mRNAs. Hence, we assessed the effect of P-body formation and active translation status of *rtt103*Δ and *rai1*Δ upon overexpression of *BFR1*. As seen in Figure 5.7A and B, overexpression of *BFR1* in *rtt103*Δ drastically reduced P-body accumulation. In *rai1*Δ, which had more cells with P-bodies at ambient temperature, the population with no P-bodies increased when *BFR1* was overexpressed. These data further hint that Bfr1 is possibly involved in the dissolution of P-bodies and effectively recycle translationally repressed mRNAs.

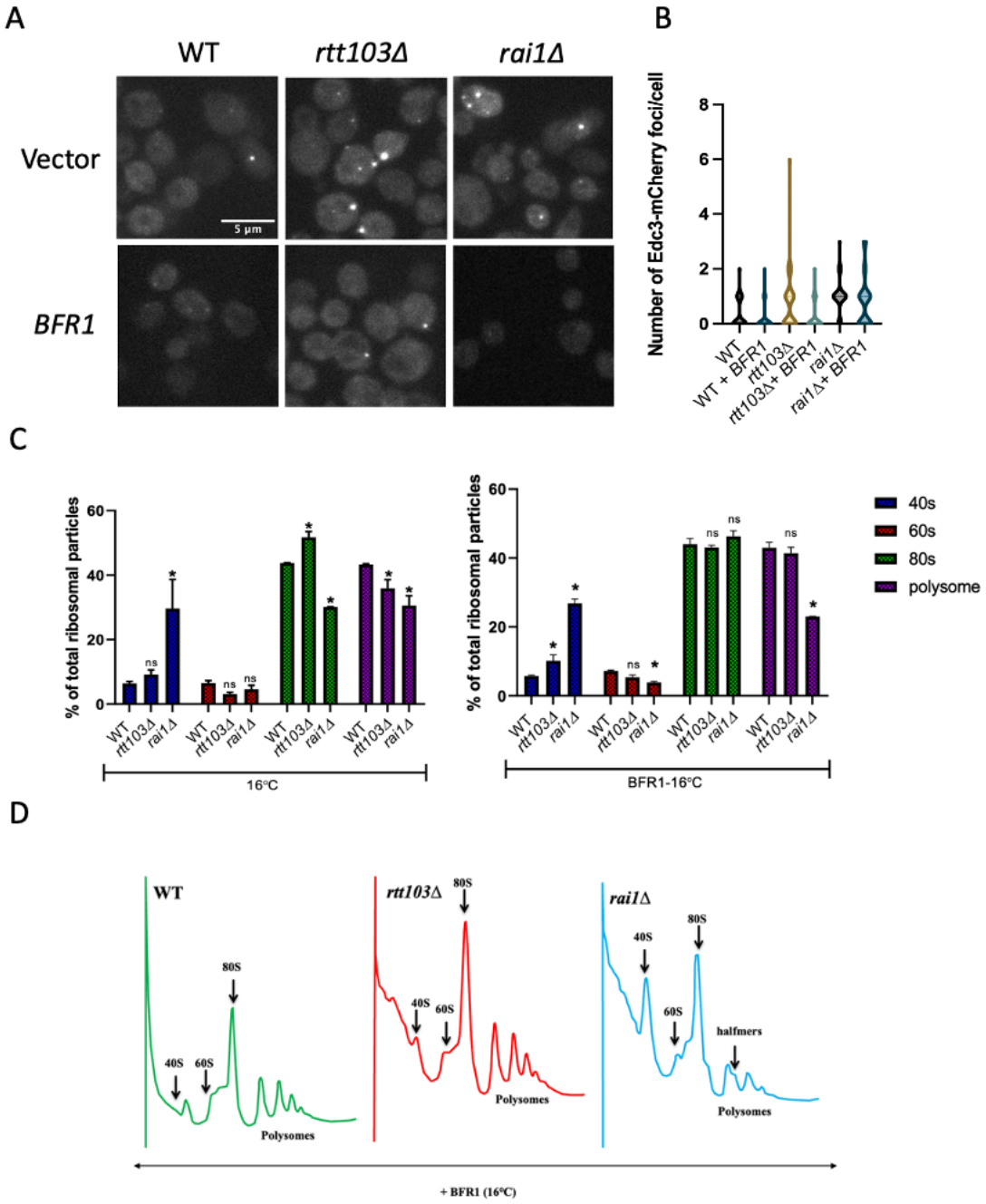

Figure 5.7: ***BFR1* overexpression rescues P-body accumulation in *rtt103*Δ and *rai1*Δ:** A. Representative images of the distribution of Edc3-mCherry foci in *rtt103*Δ *and rai1*Δ under *BFR1* overexpression. The scale bar equals 2 µm and is shown in the top left panel. B. Quantification of the number of Edc3-mCherry foci per cell (n=100). C. Polysome profile analysis of WT, *rtt103*Δ, and *rai1*Δ at 16°C upon overexpression of *BFR1*. The error bars represent the SEM and statistical significance was calculated using two-way ANOVA (*$p<0.05$). D. The optical density profiles (OD_{A254}) of the polysome gradient fractions of WT,

*rtt103*Δ, and *rai1*Δ at 16°C upon overexpression of *BFR1*. The arrows indicate individual peaks corresponding to 40S, 60S, 80S (monosomes), polysomes and halfmers.

Further, to investigate whether *BFR1* overexpression improves the overall translational status of *rtt103*Δ and *rai1*Δ, strains harbouring either *BFR1* or empty vector were subjected to polysome analysis after transient exposure (2 hours) to 16°C. As seen in Figure 5.7C, overexpression of *BFR1* restored the monosome fraction in both the mutants similar to wild type. Although the accumulation of 40S subunits still persisted in both *rtt103*Δ and *rai1*Δ, the active polysomes fraction was completely restored in *rtt103*Δ. Whereas in *rai1*Δ, the active polysome fraction was not entirely restored; however, the appearance of halfmers suggests a significant association of 43s pre-initiation complex (Figure 5.7D). Further, the percentage of cells with zero foci has significantly increased upon *BFR1* overexpression. Taking these results together, we interpret that *BFR1* overexpression does not increase the overall ribosome biogenesis *per se* but increases the effective translocation of translationally repressed mRNAs and active recycling of stalled ribosomal subunits to translate stress-specific mRNAs necessary for survival.

TSR4 and the mechanism of suppression

In *Saccharomyces cerevisiae,* processing of 35S pre-rRNA occurs both co- and post-transcriptionally, whereas in higher eukaryotes, it is exclusively post-transcriptional[183,221]. The maturation of single polycistronic precursor 35S into substrates for small subunit (18S rRNA) and large subunit (5.8S and 25S rRNA) occurs by a cascade of exo- and endo-nucleolytic reactions. Within the pre-rRNA, 18S, 5.8S, and 25S/28S are separated by ITS-1 and ITS-2 (internal transcribed spacer) with ends flanked by 5′-ETS and 3′-ETS (external transcribed spacer). The pre-rRNA co-transcriptionally associates with numerous small nucleolar ribonucleoprotein particles (snoRNPs), ribosomal proteins, and non-ribosomal proteins to stabilise its folding and to establish secondary modifications[221]. Along this process, subsequent cleavage at A_0, A_1 in the 5′-ETS and A_2 at the ITS-1 gives rise to pre-rRNA species 20S and 27S A_2. Now the production of pre-40S and pre-60S takes two alternative pathways (Figure 5.8). The maturation of 27S A_2 is restricted to the nucleolus, whereas the 20S is rapidly exported to the cytoplasm and its subsequent endonucleolytic cleavage at the D site by Nob1p

results in 18S maturation[222]. Apart from Nob1, Nsr1 and Tsr4 were also found to play a role in 18S rRNA maturation, as the mutants displayed impaired synthesis of 18S rRNA[223–225].

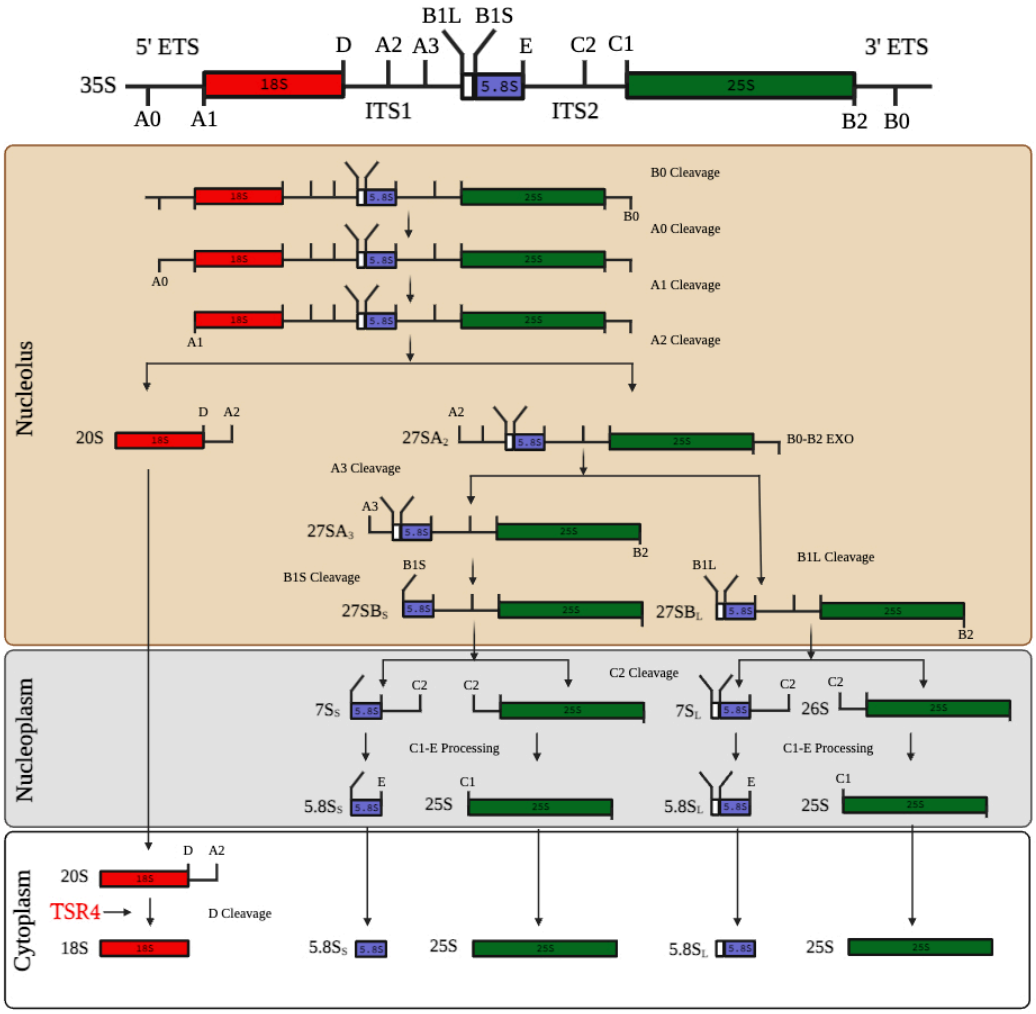

Figure 5.8: **Pre-ribosomal RNA (rRNA) processing in *S. cerevisiae*:** The pre-rRNA was initially synthesized as a long polycistronic transcript (35S) and then processed into individual mature transcripts (18S, 25S, 5.8S) by a cascade of exo- and endo-nucleolytic action spanning across three compartments. The mature transcripts are highlighted as boxes, and the internal and external transcribed regions via thin lines. Horizontal lines represent the cleavage sites with numbered letters.

Besides its role in 18S rRNA maturation, Tsr4 acts as a dedicated r-protein chaperone for ribosomal protein Rps2. It associates co-translationally with Rps2, regulating its expression

and facilitating its import into the nucleus. However, unlike other r-protein chaperones, it does not enter the nucleus, suggesting its interaction is restricted to the cytoplasm[225,226]. As the significant function of Tsr4 is the maturation of 20S to 18S, we overexpressed matured 18S ectopically under the control of RNA Pol II to assess whether it can suppress the cold sensitivity. WT, *rtt103*Δ, and *rai1*Δ harbouring GAL7-18S rDNA or empty vector were grown overnight on SC-TRP complete medium and spotted on SC-TRP raffinose or SC-TRP galactose. As seen in Figure 5.9A, overexpression of 18S rRNA suppressed the cold sensitivity exhibited by both *rtt103*Δ and *rai1*Δ. As seen for 35S rDNA expression, the suppression was more evident on the raffinose plate than on the galactose plate at 16°C. This further supports our previous observation that strong induction on galactose might be toxic as the cells have to maintain equimolar amounts of RPs to rRNA. Assessment of P-body dynamics upon overexpression of Tsr4 did not have any effect, revealing that the mechanism of suppression is quite different from that of Bfr1 (Figure 5.9B). Further, to investigate the impact of Tsr4 overexpression on the translational status of *rtt103*Δ and *rai1*Δ, strains harbouring either *TSR4* or empty vector were subjected to polysome analysis after transient exposure (2 hours) to 16°C. Overexpression of *TSR4* also exhibited a similar polysome profile as that of *BFR1* indicating both the suppressors did not improve the overall ribosome biogenesis; instead, the translationally attenuated monosome fraction was restored into active polysomes (Figure 5.9C). The accumulation of 40S subunits still persisted in both *rtt103*Δ and *rai1*Δ, whereas the active polysome fraction was restored completely only in *rtt103*Δ and not in *rai1*Δ. Further, upon overexpression there were appearance of halfmers in the polysome fraction suggesting a significant association of 43s pre-initiation complex (Figure 5.9D).

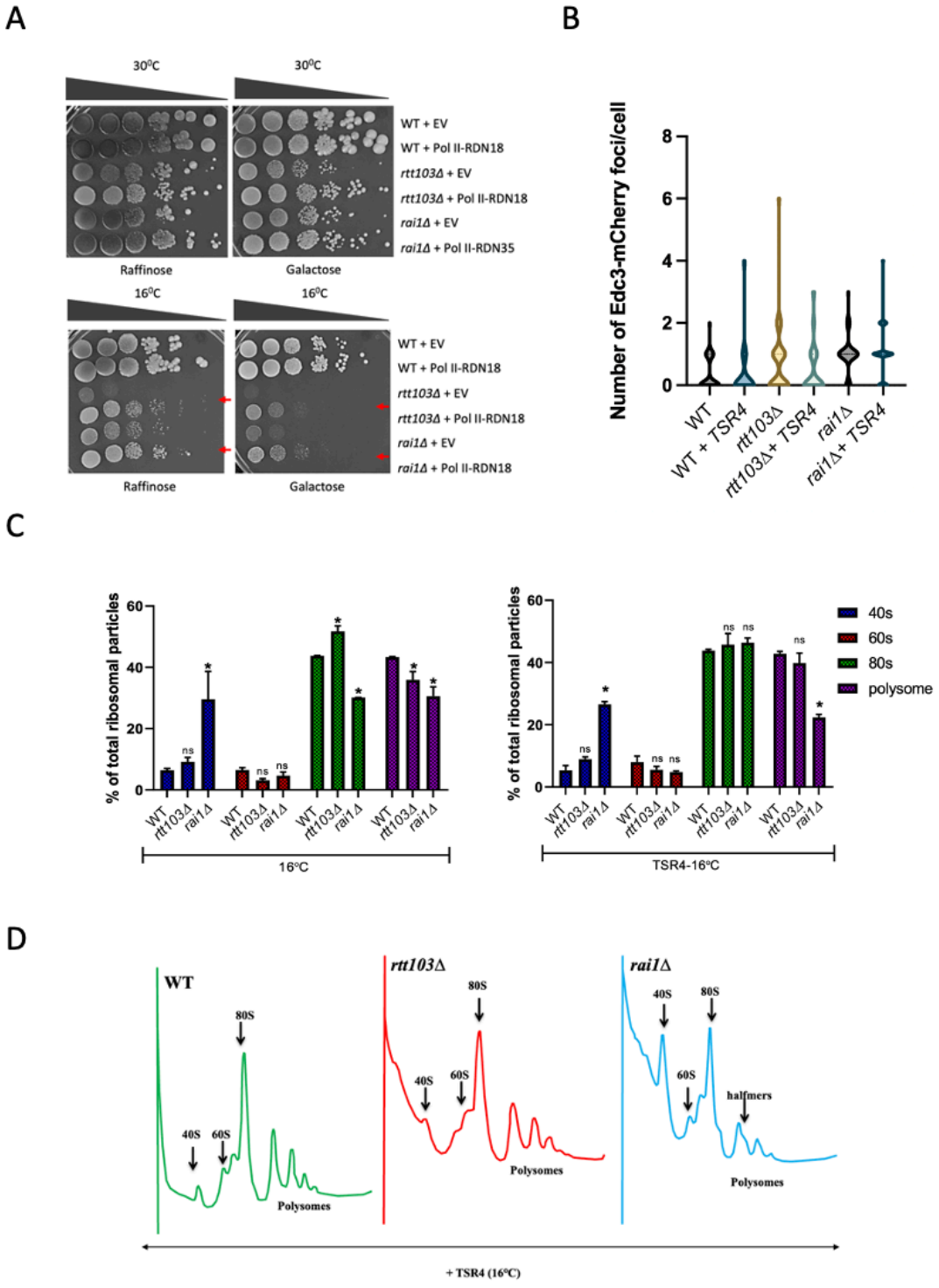

Figure 5.9: **Overexpression of RDN18 rescues cold sensitivity of *rtt103*Δ and *rai1*Δ:** A. WT, *rtt103*Δ, and *rai1*Δ strains were transformed with either empty vector or plasmids carrying RDN18 under the control of RNA Pol II driven galactose inducible promoter were grown overnight in SC-TRP broth and 5 µl of 10-fold serial dilution was spotted on SC-TRP Raffinose

and SC-TRP Galactose. The plates were imaged after five days of incubation at 30°C and 16°C. B. Quantification of the number of Edc3-mCherry foci per cell (n=100). C. Polysome profile analysis of WT, *rtt103*Δ, and *rai1*Δ at 16°C upon overexpression of *TSR4*. The error bars represent the SEM and the statistical significance was calculated using two-way ANOVA (*p<0.05). D. The optical density profiles (OD_{A254}) of the polysome gradient fractions of WT, *rtt103*Δ, and *rai1*Δ at 16°C upon overexpression of *TSR4*. The arrows indicate individual peaks corresponding to 40S, 60S, 80S (monosomes), polysomes and halfmers.

5.3 Discussion

Ribosome biosynthesis is a dynamic and energy-intensive process in eukaryotes. In an actively dividing *Saccharomyces cerevisiae*, 60% of the transcriptional activity is dedicated to the production of ribosomes[168,227]. A typical cell harbours 200,000 ribosomes, produced at a rate of ~2000 ribosomes per minute, accounting for ~50% of the total cellular protein [227]. The biogenesis requires the coordinated action of three RNA polymerases and more than 200 trans-acting assembly factors spanning three compartments[162,182]. Hence, it is not surprising that such a highly energy-intensive process is tightly regulated to maintain equimolar amounts of ribosomal proteins and rRNA. Mounting evidence suggests a complex picture for this highly regulated regulon as the cells finetune their levels depending on the type of stress and nutrient conditions[227]. In our study, we used cold sensitivity as a phenotype to uncover three more players involved in this regulation.

Studies so far in yeast have revealed that at least part of this regulation at the transcript level is achieved via some common transcription factors that bind to upstream regulatory elements of RPs. Based on the transcription factors involved, they are broadly classified into 3 categories. In category I - Hmo1 regulates the expression of RPs whose promoter is bound by Rap1–Fhl1–Ifh1 along with Sfp1. Category II has a roughly equal number of participants from Category I, but is devoid of Hmo1. Category III is a heterogenous composite of all the above-discussed players with Abf1 or Rap1 being interchangeable at some promoters. Independent of these promoter variations, their co-regulation mostly depends on the well-conserved target of rapamycin protein kinase (TOR) pathway[172,228]. Together, all the above findings describe

transcriptional regulation of ribosomal components. In our study, we have found that the absence of transcription termination complex Rtt103-Rai1-Rat1 also impedes multiple processes involved in ribosome biogenesis.

From our RNA-Seq data, we found that 60% of the genes down-regulated in *rtt103*Δ fall under the category of ribosome biogenesis (RNA Pol I/Pol III subunits/35s, pre-rRNA/ribosomal subunits/ribosomal assembly factors and chaperones) (Figure 4.6A and Table 4.9). Furthermore, *rai1*Δ also had similar expression profiles for many of the genes tested, suggesting a role of this termination complex in the regulation of ribosome biogenesis (Figure 4.8A). Polysome analysis revealed that the translation is compromised in both *rtt103*Δ and *rai1*Δ. Even under unperturbed conditions, both the mutants revealed higher monosome levels indicating translational attenuation and significant accumulation of 40S subunits. These effects were further exacerbated upon transient exposure to reduced temperature (Figure 5.3). Further, we validated the defect in ribosome biogenesis by assessing the sensitivity of mutants to varying concentrations of translational inhibitor, viz. hygromycin B (Figure 5.4). The level of sensitivity was in direct correlation with the defect in ribosome biogenesis and hence, *rai1*Δ was more sensitive even at lower concentrations.

The optimal production of pre-rRNA by RNA Pol I and III is a prerequisite and rate-limiting step in ribosome biogenesis. Several studies suggest a cross-talk among all three RNA polymerases to regulate this coordinated process.[172,205] However, the precise mechanism still remains elusive. Down-regulation of several RNAP I (4) and RNAP III (3) subunits in *rtt103*Δ and *rai1*Δ made us speculate that the downstream effects on dysregulation of ribosomal subunits, ribosomal assembly factors, and chaperones might be its consequence. Hence, we overexpressed 35S rRNA under the control of RNA Pol II and found it suppresses the cold sensitivity exhibited by *rtt103*Δ and *rai1*Δ to a significant degree (Figure 5.4).

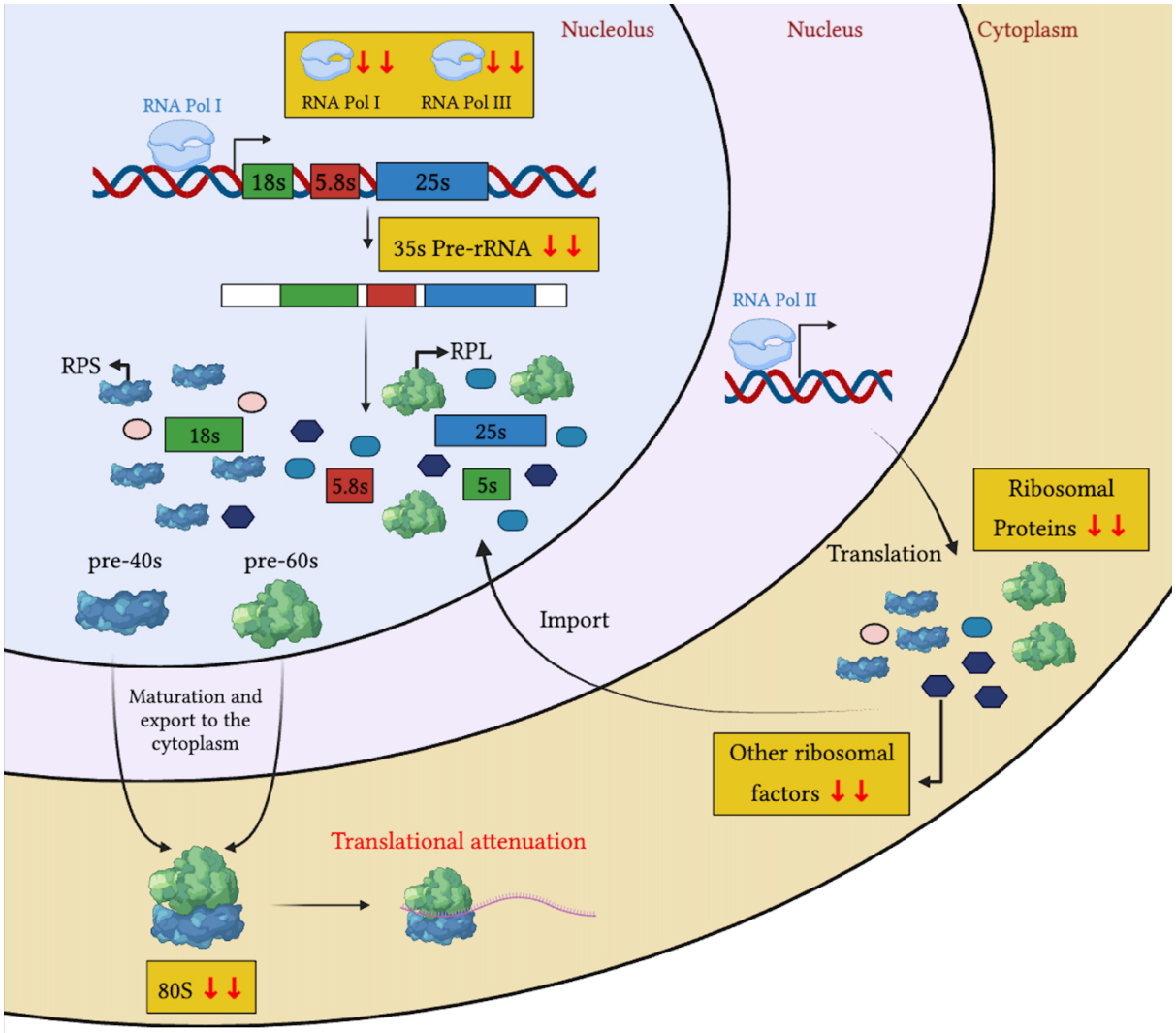

Figure 5.10: **Loss of Rtt103 leads to reduced global translation:** In summary, loss of Rtt103 results in down-regulation of several classes of RNA involved in ribosome biogenesis (Highlighted in boxes). Our results suggest that the transcription termination complex (Rtt103-Rai1-Rat1) is one of the key determinants for transcriptional regulation of ribosomal components.

The evolving new paradigm of ribosome synthesis suggests there is a heterogeneous population of ribosomes that exists, which requires the expression of RPs at different levels in different environments and even in a tissue-specific manner[229]. This suggests that many, but not all, RPs' transcription or translation may be controlled by common elements or interconnected networks. Hence, we attempted to isolate extragenic suppressors for *rtt103*Δ and *rai1*Δ cold sensitivity and found *BFR1* and *TSR4* as active suppressors. Bfr1 is a part of

Scp160 associated mRNP–polyribosome complex involved in targeting translationally repressed mRNAs into P-bodies upon prolonged stress[211,218]. From our polysome analysis, it was evident that mutants displayed a significant fraction of monosomes, indicating translational repression. We, therefore, assessed the dynamics of P-bodies upon over-expression of *BFR1*. Upon overexpression, it diminished the attenuated monosome fraction, which was evident from both our microscopic analysis of P-body dynamics and polysome profile (Figure 5.6). Although it was not a complete restoration in the case of *rai1*Δ*,* it was sufficient to suppress the cold sensitivity. The restoration effect was milder probably due to the degree of severity in ribosome biogenesis defect in comparison to *rtt103*Δ. So far, there is no direct evidence that ribosomes exist in P-bodies; therefore, we speculate that effective sequestration of translationally stalled mRNP complexes by Bfr1 into P-bodies might free the bound ribosomes on these mRNA-ribosome complexes, which can then be effectively used for translation of stress-specific mRNAs.

Tsr4 is a dedicated r-protein chaperone for ribosomal protein Rps2 and is involved in the maturation of 20S to 18S. This is the only rate-limiting step in rRNA synthesis that takes place in the cytoplasm. Further, Tsr4 localisation is also restricted to the cytoplasm[226]; hence, we overexpressed mature 18S ectopically under the control of RNA Pol II. Overexpression of 18S rescued the cold sensitivity of both *rtt103*Δ and *rai1*Δ effectively (Figure 5.8A). Further, overexpression of Tsr4 did not alter the P-body dynamics implying the mode of suppression is different from that of Bfr1 (Figure 5.8B). From polysome analysis, it was evident that Tsr4 overexpression also drastically reduced the monosome fraction and improved the active translational status (Figure 5.8C). Overexpression of neither Bfr1 nor Tsr4 increased the ribosome biogenesis *per se* as both the mutants still displayed an altered ratio of 40S/60S.

Taken together, we have identified two key players that can finetune the active translational status of the cell without improving the ribosome biogenesis as a whole. The important implication of this study is that global translational reduction can be a consequence of reduced transcription termination factors. Whether it is the termination activity of these factors or other so far unidentified function of these factors or the imperfectly terminated RNA species is not clear and awaits further analysis.

CHAPTER 6

6.1 Discussion

Transcription termination by RNAP II is a complex process requiring multiple protein factors. In a densely packed genome like that of *Saccharomyces cerevisiae*, transcription termination is valuable not only for the synthesis of the correct length of RNA but also for the efficient recycling of RNA Pol II. Improper termination or transcription read-through might also interfere with the downstream DNA-associated machineries and potentially compromise the genomic stability[33]. Hence defects in 3′-end processing machinery are associated with several disease states[230]. Much of the recent evidence highlights the importance of termination in the regulation of gene expression[231]. Regardless of its importance, termination is the least understood process in terms of regulation compared to the other stages of transcription.

In *Saccharomyces cerevisiae,* the transcription termination occurs majorly via two different mechanisms: the poly(A)- dependent pathway (Rtt103-Rai1-Rat1) for protein-coding genes and the Sen1-dependent pathway (Nrd1-Nab3-Sen1), which preferentially terminates non-coding RNA and short coding transcripts. The specificity of a particular pathway is typically determined by a combination of termination signals present on the nascent RNA molecule and the recognition of specific phosphorylation patterns on the CTD of the largest subunit of RNA Pol II[32]. Rtt103 is one such transcription termination factor that interacts with RNA pol II via its CTD domain when the CTD is phosphorylated at serine 2 of the conserved heptad repeat (Y_1-S_2-P_3-T_4-S_5-P_6-S_7). Rtt103 is an abundant nuclear protein and is conserved from yeast to mammals. It has been proposed that Rtt103 recruits Rai1 and Rat1 to RNA Pol II, where Rai1 is thought to facilitate the exonuclease activity of Rat1 during termination[47]. Rat1 is an essential protein which exhibits 5′ to 3′ exonuclease activity, whereas Rtt103 and Rai1 are non-essential for survival under optimal growth conditions.

Previous investigations regarding Rtt103 from our laboratory have reported that overexpression of Rtt103 can partially suppress the temperature and MMS sensitivity of *yku70*Δ and that the double mutants *rtt103yku70*Δ had exacerbated sensitivity to DNA

damage[81]. In this work, we have uncovered two more novel roles of this transcription termination complex (Rtt103-Rai1-Rat1) in telomere metabolism and ribosome biogenesis[202].

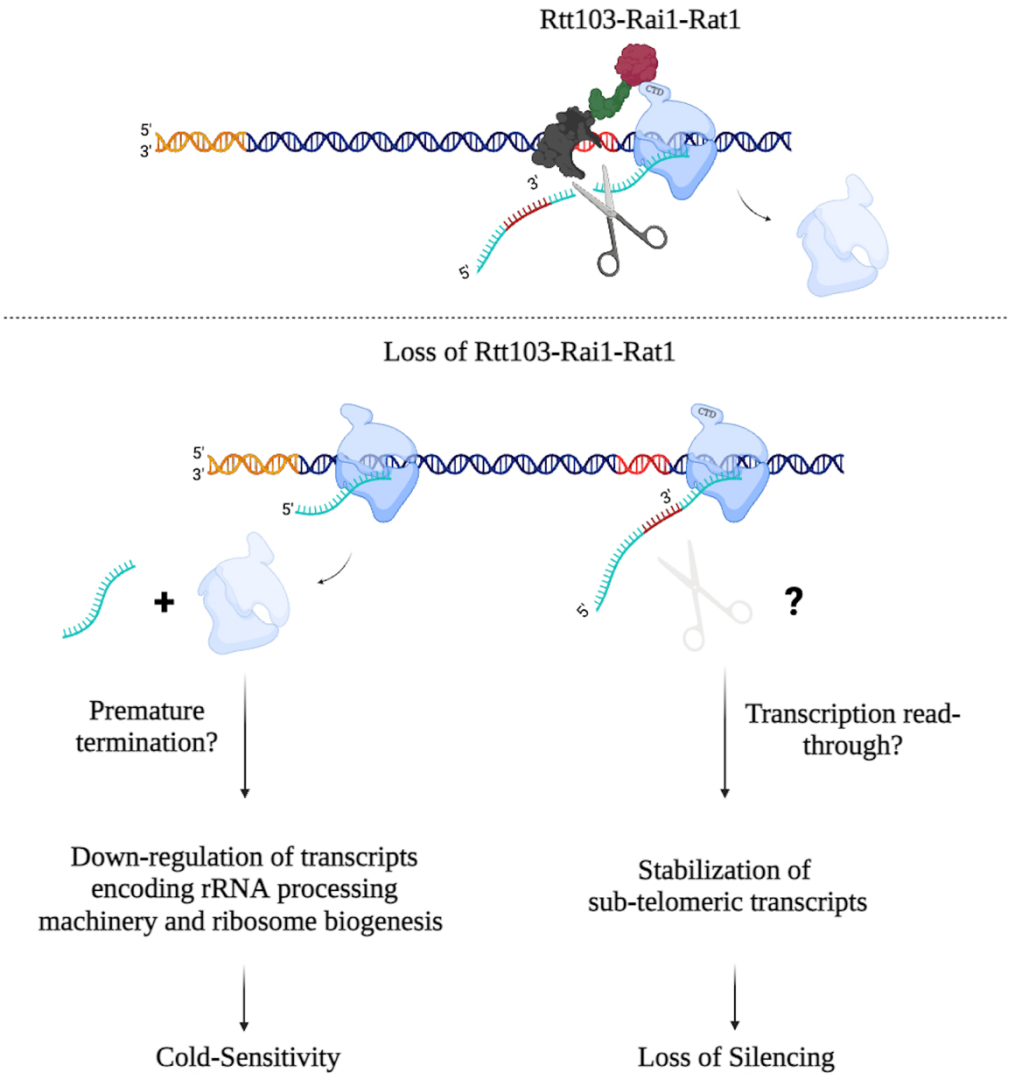

Figure 6.1: **Transcription termination defines the fate of the transcript:** In summary, we speculate that loss of transcription termination complex (Rtt103-Rai1-Rat1) might result in either premature termination in case of genes encoding for ribosomal components or read-through transcripts in case of sub-telomeric transcripts, thereby determines the stability of the resulting transcript.

"Terminators at the Terminus": Transcription Termination complex Rtt103-Rai1-Rat1 regulates sub-telomeric transcripts in *Saccharomyces cerevisiae*

In *Saccharomyces cerevisiae,* three heterochromatic loci are subjected to gene silencing: the telomeres, silent mating-type loci, and rDNA repeats. The RNA Pol II machinery is inaccessible to these loci due to the higher order of repressive chromatin structures, cis-acting regulatory sequences, and protein structural components associated with these loci[232,233]. The yeast Yku complex (Yku70-80) is one such structural component associated with the termini of the telomeres and performs multiple functions. Loss of either subunit results in complete loss of silencing at the telomeres (TPE)[124,234]. Hence, we assessed whether Rtt103 plays any role in telomere metabolism or gene silencing. In this work, we established that Rtt103 is required for efficient silencing of telomeric and sub-telomeric transcripts. Similar to its role in transcription termination, the entire complex (Rtt103-Rai-Rat1) is involved in this regulation. Further, we showed that it is not the exacerbated transcription rate in the absence of this complex but rather, it is the stability of sub-telomeric transcripts that is specifically altered[202].

Telomere dysfunction is linked to several human diseases, including cancer and ageing[92]. Hence, the cells maintain a critical length for proper telomere homeostasis and to prevent premature senescence[90]. The cells usually employ two different mechanisms to lengthen the critically shortened telomeres: addition of telomeric repeats by ribonucleoprotein enzyme telomerase or alternative lengthening of telomeres via homology-dependent recombination mechanism[90,91]. Recent evidence suggests that telomere length and telomerase activity are regulated by TERRA. Altered expression of TERRA is associated with changes in telomere length and promotes genome instability and cellular senescence[110]. Studies suggest that TERRA expression is tightly regulated via Rap1p and Rat1p-dependent mechanism with a minor contribution from other RNA surveillance pathways. However, the exact mechanism and regulation were not known.

In our study, we provide evidence that these transcripts are regulated in a transcription-dependent manner and that Rtt103-Rai1-Rat1 is physically associated at the telomeres. We show that the recruitment of exonuclease Rat1 to the terminus is dependent on Rtt103 in a transcription-dependent manner. Inhibition of transcription or abolishing Rtt103 interaction

with RNA Pol II significantly reduced Rat1 association at the telomeres. This provides a mechanistic basis for how Rat1 regulates TERRA levels. As TERRA lacks the conserved polyadenylation and cleavage signal, the exact mechanism of termination was not known. Our work hints that the transcription termination mechanism might be similar to that of protein-coding genes by Rtt103-Rai1-Rat1 and regulates its stability[202].

The sub-telomeric regions reveal higher levels of recombination leading to faster evolution of gene families residing in this locus[235–237]. This allows faster and better adaptive responses to the changing environment. Additionally, it contributes to antigenic variation and virulence in some pathogenic yeasts and parasites[105,238–240]. Therefore, understanding the regulation of telomeric and sub-telomeric transcription has implications beyond yeast. While this study shows Rtt103-Rai1-Rat1 termination complex is involved in such regulation, a study of biological cues that decide between maturation or co-transcriptional degradation of sub-telomeric transcripts might provide practical ways to inhibit transcription of genes that are sub-telomeric in origin. The sub-telomeric region also harbours various other classes of genes like *tlh,* which is regulated under nitrogen starvation, *cri-TER*, a temperature-dependent non-coding RNA, and genes responsible for biofilm formation[241–243]. It remains to be investigated if the expression of these genes is also regulated co-transcriptionally. Furthermore, unravelling the molecular mechanisms underlying this regulation would open up new possibilities for improving treatment strategies against various age-related and parasitic infections.

Our study raises a number of questions to be addressed. Induction or overexpression of TERRA from a single telomere induces early-onset senescence[244]. Does stabilization of TERRA also have similar effects? Is transcription of TERRA a coordinated event from all the telomeres? In the case of sub-TERRA and ARIA, it is transcribed from telomere end towards the centromere. Do the telomere ends also possess promoter-like elements? In *S. cerevisiae*, sub-TERRA XUT and sub-TERRA CUT are complementary to each other and can form dsRNA. As RNAi does not exist in *S. cerevisiae*, could RNA degradation pathways be more critical to regulate the levels of such non-coding telomere transcripts?

A Novel Role of Transcription Termination complex Rtt103-Rai1-Rat1 in Ribosome Biogenesis

Ribosome biogenesis is a complex and energy-intensive process. It requires an orchestrated transcriptional output of hundreds of genes. It acts as a direct measure of cell growth potential and is regulated in accordance with the cellular energy status or environmental cues. Based on the available data, we deduce that there might be a direct correlation between cold sensitivity and ribosome biogenesis. We found that loss of transcription termination factors Rtt103, Rai1, or Rat1 confers cold sensitivity. In our work, when we isolated suppressors of this cold sensitivity, we obtained 2 suppressors that are linked to ribosomes. In addition, when we expressed rRNA under the control of RNA Pol II, it effectively suppressed cold sensitivity. Bfr1 and Tsr4, the two extragenic suppressors obtained, effectively suppressed the cold sensitivity and improved overall translation without increasing ribosome biogenesis as a whole.

In *rtt103*Δ*,* even under unperturbed conditions, 60% of the downregulated genes belong to the category of rRNA processing. This includes several notable critical players in ribosome biogenesis, including subunits of RNA Pol I, RNA Pol III, rRNA, ribosomal proteins, Nops, ribosomal chaperones, and assembly factors (Figure 5.10). As these mutants already have reduced levels of components involved in ribosome biogenesis, the increased biogenesis demand under cold temperatures may not be met in these mutants. Taken together with our suppressor results, this indicates that the cold sensitivity exhibited by these mutants was due to defects in ribosome biogenesis, which is a hitherto unreported phenotype for the Rat1-Rai1-Rtt103 complex.

In *Saccharomyces cerevisiae,* ribosome biogenesis is principally regulated at the transcription level followed by mRNA maturation[168]. Recent evidence suggests that RNA itself can act as a significant regulator of the regulon via differential splicing, alternative transcription termination, or differential translation of RP paralogs. It was shown that the transcript of the large ribosomal subunit protein RPL9B can terminate via two alternative pathways depending on the Rpl9p levels[35]. When Rpl9p is in excess, it binds to the stem-loop structure at the 3′UTR and negatively impacts the termination via Rtt103-Rai1-Rat1. This preferentially aids the termination via the alternative pathway Nrd1-Nab3-Sen1 and targets them for exosome-

mediated degradation. In the case of RPL8A, the choice of termination pathway depends on growth conditions and exposure to stress[36]. RPS14B and RPL30 are regulated at the level of pre-mRNA splicing[245,246], whereas RPS3, RPL4A, and RPS28B are autoregulated at the level of mRNA decay[247–249]. Further, in mammals, it has been shown that the 3′-UTR dictates the localisation of RP mRNA and regulates its expression[250,251]. Hence, we speculate it might be transcription read-through or aberrant transcription termination in these mutants that specifically alters the stability of the downregulated transcripts. Additionally, 90% of the mRNA splicing events in *Saccharomyces cerevisiae* occur on the RP encoding mRNAs[168]. Rat1, bearing direct interaction with some of the splicing proteins, and its involvement in co-transcriptional splicing of RP encoding mRNA, suggests that it might also be a possible cause for the downregulation of selective transcripts in the absence of this complex[63].

This study has raised a number of questions that remain to be addressed to understand the interconnected networks and common mechanisms that regulate the ribosome biogenesis regulon. Overexpression of *BFR1* effectively rescued *rtt103*Δ translational efficiency, wherein multiple factors associated with ribosome biogenesis were down-regulated. From the polysome analysis, it was quite evident that overexpression did not improve the overall ribosome biogenesis; rather, the translationally attenuated monosome fraction was restored into active polysomes. The P-body dynamics hinted that the mechanism of suppression might be via the effective sequestration of translationally repressed mRNP complexes and recycling of attenuated ribosomes. It would be insightful to overexpress the mutant variant of *BFR1* F239A – which is devoid of RNA binding to validate the above speculation further. In the case of *TSR4* overexpression, owing to its already-established functions of 18s rRNA processing and chaperonin activity of Rps2, looking at the processing of 20s rRNA or overexpression of *RPS2* in the mutants might shed light on the exact mechanistic details of the suppression. Further, it will be interesting to see whether overexpression of these suppressors can also improve the survivability of other mutants with reduced translational efficiency?

Our work has highlighted the significance of transcription termination factors Rtt103-Rai1-Rat1 role in maintaining global translation levels. The regulation of gene expression at the level of transcription termination needs further exploration to derive the consequences of improper termination. It appears that improper termination may lead to decreased transcript levels as in the case of ribosome biogenesis components or increased stability as in the case of

sub-telomeric transcripts. What is the mechanistic link between transcript stability and transcription termination?

Finally, understanding the global regulation of ribosome biogenesis has important consequences for healthy and disease states. Ribosomopathies are a group of diverse, rare disorders caused by ribosomal dysfunction[252]. While all of them have some defect in ribosomal function, they have specific phenotypic consequences, including tissue-specific differences. In humans, there are cases where mutations in any one of the ~14 RPs result in impaired bone marrow function (Diamond-Blackfan Anemia). Defects in rRNA processing have been suggested to play a role in the X-linked form of Dyskeratosis Congenita, which is caused by mutations in the *DKC1* gene that encodes an enzyme for pseudouridylation of RNA, loss of which leads to impaired translation. The tissue-specific effect of these and other mutations leading to ribosomapathies are attributed to differential function of ribosomes in different tissues and during different stages of development. Can transcription termination defects also lead to ribosomapathies? Importantly, as suggested by this work, can improving global translation efficiencies address the defects in ribosome biogenesis defects of ribosomapathies?

Over the years, several studies have highlighted the importance of transcription initiation and elongation in spatiotemporal gene regulation. However, the genome-wide implication of proper transcription termination remains an unexplored area in the context of gene regulation. The absence of proper termination results in the generation of premature or aberrant read-through transcripts, which poses a serious threat to genome stability and can have multiple other consequences. Hence, future research should focus more on understanding the mechanistic basis of transcription termination to unravel the role of termination in the regulation of diverse cellular processes.

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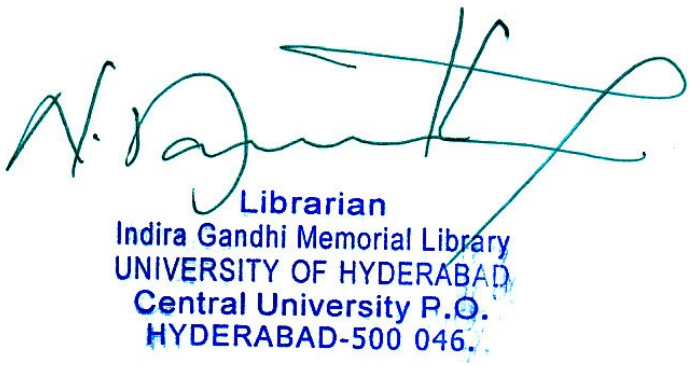

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Role Of Transcription Termination Factors, Rtt103-Rai1-Rat1, in Gene Regulation

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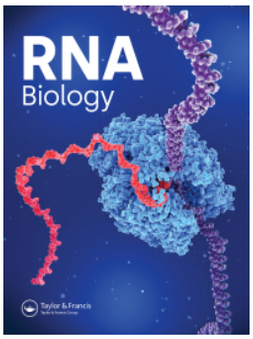

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Transcription termination complex, Rtt103-Rai1-Rat1, regulates sub-telomeric transcripts in *Saccharomyces cerevisiae*

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ABSTRACT

Telomeres are terminal structures that define the ends of linear chromosomes. They harbour specialized ribonucleoprotein complexes which play a major role in genome integrity by preventing unscheduled DNA damage repair events. Genes located adjacent to telomere repeat sequences are repressed by a phenomenon called telomere position effect (TPE) via epigenetic silencing. RNA surveillance pathways post-transcriptionally regulate any leaky transcripts arising from the telomeres. Recently, multiple non-coding RNA species originate from telomere ends, namely, TERRA (telomeric repeat-containing RNA), ARRET, sub-telomeric XUTs and sub-telomeric CUTs have been identified. In this study, we report a role for the transcription termination complex (Rtt103-Rai1-Rat1) in regulating the abundance of the sub-telomeric transcripts in a transcription-dependent manner. We show that the Rtt103 mutants have elevated levels of TERRA and other sub-telomeric transcripts that are usually silenced. Our study suggests that Rtt103 potentially recruits the exonuclease, Rat1 in a RNA polymerase II dependent manner to degrade these transcripts and regulate their levels in the cell.

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Introduction

Telomeres, the terminal structure of eukaryotic linear chromosomes, are an array of specialized nucleoprotein complexes. They play a crucial role in maintaining genome integrity by protecting the ends from being recognized as DNA-double strand breaks (DSBs) [1]. Like most organisms, the chromosome ends of *Saccharomyces cerevisiae* consist of a short array of tandem repeats ($C_{1-3}A/TG_{1-3}$) that averages approximately 300 ± 75 bp in size [2]. The ends have three well-defined regions, namely, sub-telomeric, middle and repetitive elements, often referred to as TAS elements (Telomere-Associated Sequences). TAS are of two classes, namely, X and Y'. The Y′ elements are either long (6.7 kb) or short (5.2 kb) and are present in 0–4 copies per chromosome end [3]. Y' is present in only half of the telomeres, whereas X-element is present at all the telomeres. X-element is much more heterogeneous in both sequence and size than Y' elements. The sub-telomeric regions are highly dynamic and undergo frequent recombination events [4,5]. As a result, they vary significantly in size between closely related strains. Because of the dynamic nature of the TAS elements, the proteins binding to them vary from telomere to telomere and confer distinct functions [6]. The X-elements are generally transcriptionally repressed and devoid of nucleosomes. Y' elements, on the other hand, are transcriptionally active and contain nucleosomes. Furthermore, Y' elements do not exhibit the classic characteristics of heterochromatin such as high occupancy of Sir3 and Rap1, and display low levels of histone H4 lysine 16 acetylation [6].

Telomeres are subjected to continuous attrition with each cell division because of the end replication problem [7]. This is counteracted by the action of telomerase, a reverse-transcriptase which utilizes a RNA moiety as a template to lengthen the telomere ends [8]. In the absence of telomerase, the telomeres reach a critically short length after a few cell divisions and undergo an irreversible cell cycle arrest called replicative senescence [9]. In yeast, cells undergoing premature senescence use either amplification of Y' (Type I), TG_{1-3} repeats (Type II) or TERRA (Telomere Repeat containing RNA) transcripts to lengthen the critically shortened telomeres [10–13].

Another salient feature of the telomeres is the establishment of heterochromatin region. The telomeric tract is non-nucleosomal and has a distinct set of telomere specific proteins [2,14]. The major constituent is the Rap1p which binds DNA in a sequence-specific manner and recruits other proteins, namely, Rif1, Rif2 and SIR complex, which together regulate telomere length and transcription of sub-telomeric genes [15,16]. The adjacent sub-telomere sequence has nucleosomes [14]. Rap1 recruits the Silent information regulator proteins, Sir3 and Sir4 to the telomeres which in turn recruit the NAD-dependent histone acetylase that deacetylates H3 and H4 [16,17]. The association of the Sir proteins and the consequent deacetylation of nucleosomal histones represses transcription in this region [18]. Several additional factors contribute to TPE like chromatin remodellers, chromatin assembly factors, telomere resident proteins (Rif1,

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Rif2, Yku70/80), telomere folding, insulators and silencing elements at the sub-telomeres and the anchoring of the telomere to the nuclear periphery [18–24]. Silencing initiated from the telomeric tracts is discontinuous and while the X element is effectively silenced in a Sir protein-dependent manner, the Y elements are not [25]. In addition, based on genome-wide transcript analysis, it is clear that only a few genes on a few chromosomes beyond 3.5kb from the telomere tracts are silenced in a Sir protein-dependent manner, while a majority are not. However, the overall levels of transcripts from this region is low and it is not clear if it is due to reduced transcription [26].

Recently, it has become clear that despite the epigenetic silencing of sub-telomeric regions, several RNA species arise from the telomeric and sub-telomeric region. These include TERRA (telomeric repeat-containing RNA), sub-TERRA, ARRET, ARIA, sub-telomeric XUTs, sub-telomeric CUTs and several other RNA species which originate from telomere ends [27,28]. Some of these RNA species, including TERRA, are possibly universally conserved and have been detected in several organisms including yeasts, plants, parasites and mammals. The current view suggests a strong role for TERRA to be a part of the telomeric architecture as they remain physically associated with the telomeres [29,30]. Expression of TERRA is cell cycle regulated and ideal levels of TERRA are crucial for the cellular fitness as either overexpression or downregulation causes telomere dysfunction-induced foci [12,29,31–35].

In *S. cerevisiae*, TERRA is an RNA Pol II product which is regulated both epigenetically [36] and post-transcriptionally via RNA surveillance pathways [27,30]. In X-only containing telomeres, the repression of TERRA is mediated by both Sir2/3/4 and Rif1/Rif2-Rap1 complex. On the other hand in Y' containing telomeres, the repression is mediated by primarily Rap1- Rif complex [36]. The turnover of TERRA, irrespective of its origin, is mainly mediated by Rat1, a nuclear 5'-3' exonuclease [30]. But the exact molecular mechanism of TERRA turnover still remains elusive.

The transcription cycle of RNA Pol II is a highly coordinated event which is regulated via its C-Terminal Domain (CTD) [37]. In yeast, the CTD comprises 23 consecutive hexapeptide repeats (Y_1-S_2-P_3-T_4-S_5-P_6-S_7) that are dynamically phosphorylated and dephosphorylated [38]. At the promoter, the CTD mostly remains unphosphorylated [39]. As it transverses through the gene body, Ser5 phosphorylation (Ser5P) dominates and aids in the recruitment and activation of 5′- capping enzymes [40,41]. Ser5P gradually decreases as RNA Pol II progresses towards the elongation phase and phosphorylation of Ser2 (Ser2P) increases [39]. Towards the 3'end, Ser2P dominates and is involved in the recruitment of polyadenylation, cleavage and termination factors [42]. Rtt103p is one such transcription termination factor which interacts with the Ser2P RNA Pol II via its CTD interacting Domain (CID) and is thought to cooperatively recruit Rai1 and Rat1 to the 3' end of the gene body, promoting termination of transcription [43]. Apart from termination of transcription of mRNA, Rat1 and Rai1 are involved in processing the pre-rRNA transcripts [44–47].

A genome-wide distribution analysis of RNA Pol II revealed its enrichment in various 'epigenetically silenced' regions of the genome like the telomeres, rDNA 'E-Pro' region, centromeres, HML and HMR loci. Interestingly, it was shown that the transcriptional regulation at the rDNA is via the alternative transcription termination pathway Nrd1-Nab3-Sen1 [48,49]. The non-coding RNA (IGS1-R) arising from the rDNA is proposed to recruit Nrd1-Nab3-Sen1, which in turn regulates the levels of these transcripts in conjunction with TRAMP and exosome complex [49]. Furthermore a single amino acid substitution in Sen1 (E1597K) resulted in aberrant synthesis of many regulatory noncoding RNAs and mRNAs [48]. These studies suggest that there might be a fine tuning of transcript levels by further action of the transcription termination factors.

In our study, we report a novel role of transcription termination complex Rtt103, Rai1 and Rat1 in sub-telomeric gene silencing and in the regulation of the long non-coding RNAs arising from the sub-telomeric and telomeric tracts. Further, we demonstrate that this complex is physically enriched at the telomeres. The recruitment of the Rat1 exonuclease to the terminus is dependent on Rtt103 and requires active transcription suggesting a potential mechanism for the enrichment of this complex at the telomeres.

Results

rtt103Δ mutants are defective in sub-telomeric silencing

Previous investigation in *S. cerevisiae* to identify suppressors for *yku70*Δ temperature-sensitive phenotype yielded high copy expression of *RTT103* as a partial suppressor for temperature sensitivity [50]. Although we found *RTT103* does not have a role in repairing non-chromosomal substrates via non-homologous end joining (data not shown), a key function of Yku70, we extended the study to test whether Rtt103 has any role in telomere metabolism and gene silencing, another important function of Yku70. Transcription of genes located adjacent to telomeres is usually repressed by the telomere position effect (TPE) [19]. To assess the impact of Rtt103 in sub-telomeric silencing, we generated *rtt103Δ* in a strain that harbours *URA3* at the telomeric region of chromosome VIIL. The expression of *URA3* in this locus is silenced by TPE [51]. The measurement of loss of silencing was performed by spotting overnight grown cultures on the agar plates containing 5-fluoroorotic acid (5-FOA) in growth medium. The *URA3* gene encodes for an enzyme orotidine-5'-phosphate decarboxylase, which converts the 5-FOA into a toxic substance 5' fluorouridine monophosphate. Hence, loss in silencing restricts the growth of the cell in 5-FOA, and conversely, robust growth on plates containing 5-FOA indicates silencing. Wild-type cells exhibit TPE. As silencing at the telomeres is stochastic, wild-type cells grow on both SC-URA and SC + 5-FOA plates. But in an *rtt103Δ*, we found reduced silencing, as indicated by the reduction in growth on 5-FOA plate, compared to wild type (Figure 1A). *yku70Δ*, which is known to have strong silencing defects, was used a positive control and is severely defective for growth on 5-FOA plates. Silencing was restored upon complementation with either single copy (CEN) or multicopy (2 micron) *RTT103* (Figure 1A).

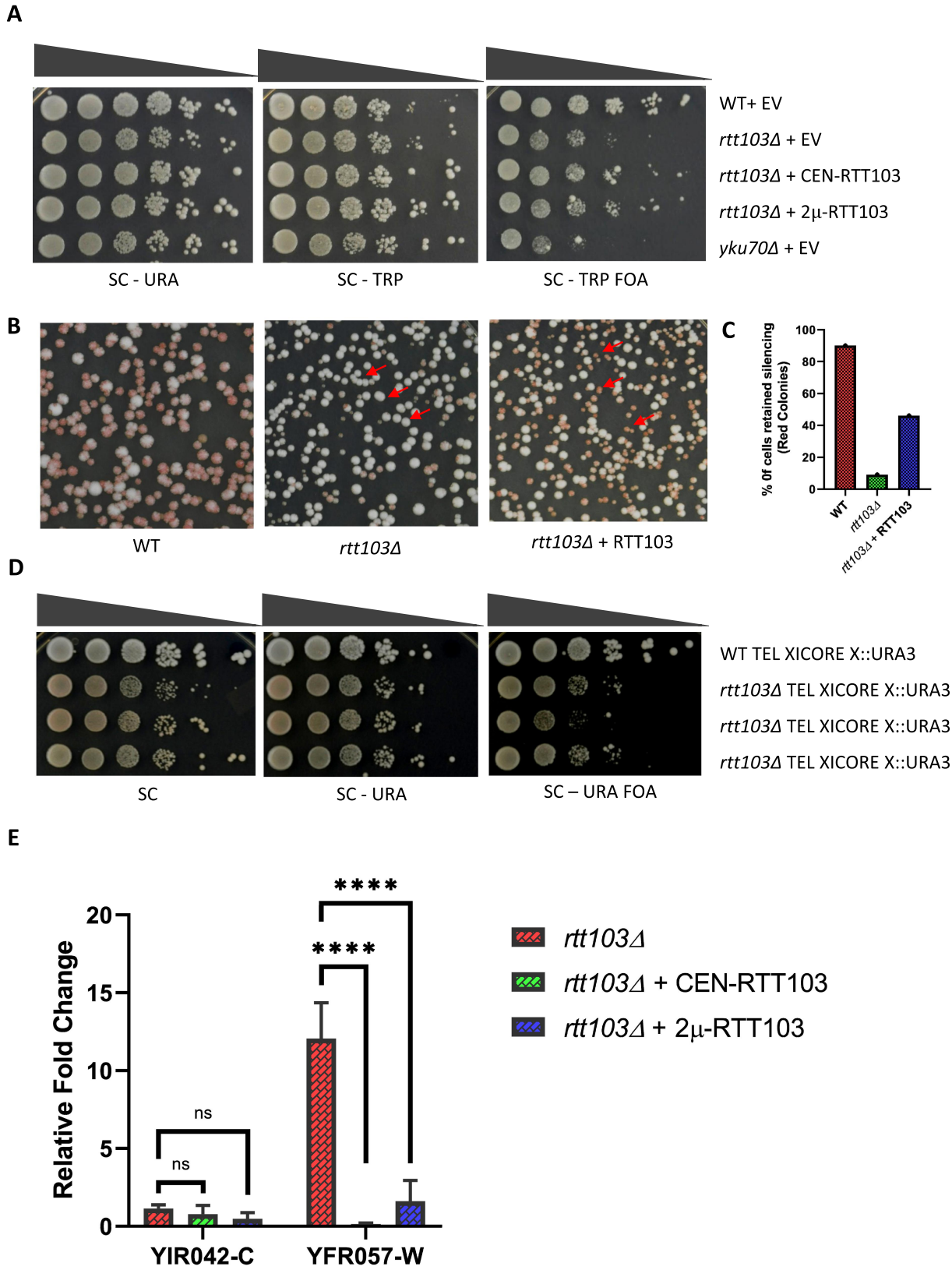

Figure 1. *rtt103Δ* is defective in sub-telomeric silencing.

Note: A. Indicated strains transformed with either empty vector (EV) or full length Rtt103 encoded on a single copy CEN vector (CEN) or multicopy 2 micron vector (2 μ) were grown overnight and 5-μl of tenfold serial dilutions were spotted on SC-TRP, SC-TRP URA and SC- TRP+FOA plates. Plates were imaged after 72 hours of growth at 30°C.

B. Wild type, *rtt103Δ* and *rtt103Δ* carrying full length Rtt103 in CEN vector were grown overnight in synthetic complete media, serially diluted tenfold and 100 μl was plated on YPD. Plates were imaged after 72 hours of growth at 30°C.

C. Bar graphs depicting the percentage of cells that retained silencing (red and red sectored colonies) has been quantified (n = 500).

D. WT and *rtt103Δ* harbouring TEL XICORE X:URA3 were grown overnight in synthetic complete media, serially diluted tenfold and 5 μl was spotted on SC, SC-URA and SC-FOA. Plates were imaged after 72 hours of growth at 30°C.

E. RNA was isolated from the indicated strains and converted to cDNA. cDNA was then subjected to qRT-PCR to assess the relative expression of YIR042-C and YFR057-W. All data are depicted as mean + SEM, n = 3. P values were obtained from two-way ANOVA.

Further, we used a colour-based assay to study the loss of silencing by integrating the *ADE2* marker at the telomeric region of chromosome VIIL. *ade2Δ* cells display a red colony colour phenotype on rich yeast growth medium, YPD. The red colour is due to the accumulation of intermediate metabolites of the adenine biosynthesis pathway. When *ADE2* is inserted proximal to the telomere sequences, wild type cells exhibit TPE and do not continuously express *ADE2*. So they appear red like an *ade2Δ* mutant, stochastically switching to white for a few generations, leading to the appearance colonies with red and white sectors (Figure 1b first plate). In an *rtt103Δ*, due to loss of silencing, 90% of the cells appeared white with no red sectors (Figure 1B,C). We noticed that in this case that the complementation with plasmid encoded Rtt103 is partial. We also generated a *rtt103Δrai1Δ* double mutant in the telomere VIIL ADE2 background and found that the silencing defect was even more severe and almost no red sectors were seen (Figure S1A). However, this strain was extremely sick and was not used for further studies.

Several studies suggest discrepancies in the silencing levels between natural telomeres and modified truncated telomeres [25]. TPE was initially studied by placing either *URA3* or *ADE2* gene immediately adjacent to the telomeric TG_{1-3} tract by removing the adjacent X and Y′ elements [52]. Moreover, TPE varies substantially from telomere to telomere in the same cell and between different strain backgrounds. It is mainly due to the difference in sub-telomeric structures at each telomere and the differential occupancy of silencing proteins in X and Y' elements [23,36,53]. Although Y' elements have high nucleosome density, they are transcriptionally active. They lack the classical hallmarks of heterochromatin, such as high Sir3 and Rap1 occupancy as well as low levels of histone H4 lysine 16 acetylation [6,18,54]. Therefore, to confirm whether the silencing defect observed in *rtt103Δ* is not specific to modified truncated telomeres, we also assessed the silencing defect of *URA3* marker inserted at the core X of chromosome XI-L of unmodified natural telomeres [55]. As shown in (Figure 1D), *rtt103Δ* strain has lowered growth in 5-FOA while growing robustly on SC-URA plates, indicating reduced silencing at the native telomeres as well. In order to further validate the silencing defect in *rtt103Δ*, we also assessed the relative levels of two native sub-telomeric genes namely *YIR042-C* and *YFR057-W* via quantitative reverse transcriptase PCR (qRT-PCR) [56]. *YIR042-C* is located at chromosome IX and 3.9Kb away from the telomere end, whereas *YFR057-W* is located at chromosome VI at 645bp away from the end and have been shown to be silenced in a Sir-protein-dependent manner [26]. qRT-PCR results revealed that in an *rtt103Δ*, there was a 12-fold increase in the relative amounts of *YFR057-W* in comparison with WT. We could only detect a modest increase in YIR042-C transcripts that was not statistically significant (Figure 1E). Complementation with either a single copy (CEN) or multicopy (2μ) *RTT103* restored silencing completely. Taken together, these data establish that silencing at the sub-telomeres is compromised in *rtt103Δ* compared to wild type.

rtt103Δ, rai1Δ *and* rat1–1 *are defective in sub-telomeric silencing*

As Rtt103 is proposed to work in concordance with Rat1 and Rai1 in transcription termination, we decided to test the role of this complex in telomere silencing. We assessed the level of silencing defect in *rai1Δ* and *rat1–1* at the two native sub-telomeric genes, namely, YIR042-C and YFR057-W, via quantitative reverse-transcriptase PCR (qRT-PCR). Rat1 is an essential gene and the *rat1–1* allele is temperature sensitive. Therefore, the silencing was assessed at 39°C in *rat1–1*. All three mutants revealed a varying range of silencing defects for YFR057-W in comparison with wild type (Figure 2A) with *rai1Δ* showing moderate increase in transcript abundance. *SIR2*, which is directly involved in epigenetic silencing of sub-telomeric loci, was used as a positive control. Again, YIR042 transcript levels were increased slightly in *rtt103Δ* and *rai1Δ* but showed a more pronounced increase in *rat1–1* and *sir2Δ*. As loss of all three proteins leads to increased accumulation of telomeric and sub-telomeric transcripts, it is possible that Rat1, Rai1 and Rtt103 together have a role at the telomeres as in transcription termination.

rtt103Δ *alters the stability of sub-telomeric transcripts*

From the existing literature, it is evident that sub-telomeric transcripts are regulated epigenetically [19], transcriptionally [30] and post-transcriptionally via RNA surveillance pathways [27,57]. In order to address whether it is exacerbated transcription rate or stability of the sub-telomeric transcripts that is altered in an *rtt103Δ*, we employed a temperature-sensitive allele of the RNA Pol II subunit, *rpb1–1*. The transcription can be turned off by inactivating RNA Pol II after shifting from permissive (25°C) to non-permissive temperature (39°C), whereas RNA Pol I and Pol III transcription status remains unaltered. We analysed the stability of transcripts arising from *YFR057-W* and *YIR042-C* along with *ACT1* at regular intervals after inhibiting transcription. Total RNA was isolated from samples harvested at 0 minutes (just before the temperature shift) and every half an hour after the temperature shift to 39°C. Relative abundance of sub-telomeric transcripts and *ACT1* transcripts was obtained by normalization to 7S RNA and plotted as a function of time. 7S RNA is a non-coding RNA arising from the *SCR1* locus, is transcribed by RNA polymerase III, and is unlikely to be affected by defective RNA polymerase II function at higher temperatures. In comparison with wild type (*rpb1–1*), *rtt103Δ rpb1–1* revealed greater stability of sub-telomeric transcripts, whereas stability of *ACT1* transcript remained the same in both (Figure2B and 2C). These data suggest that in an *rtt103Δ* mutant, it is the stability of the transcript, not the rate of transcription, which is altered and it is also specific to sub-telomeric transcripts.

TERRA accumulates in an rtt103Δ, rai1Δ and rat1–1

Recent discoveries of regulatory ncRNAs controlling cis and trans gene silencing in budding yeast have led to speculation that these RNAs might be directly involved in heterochromatin

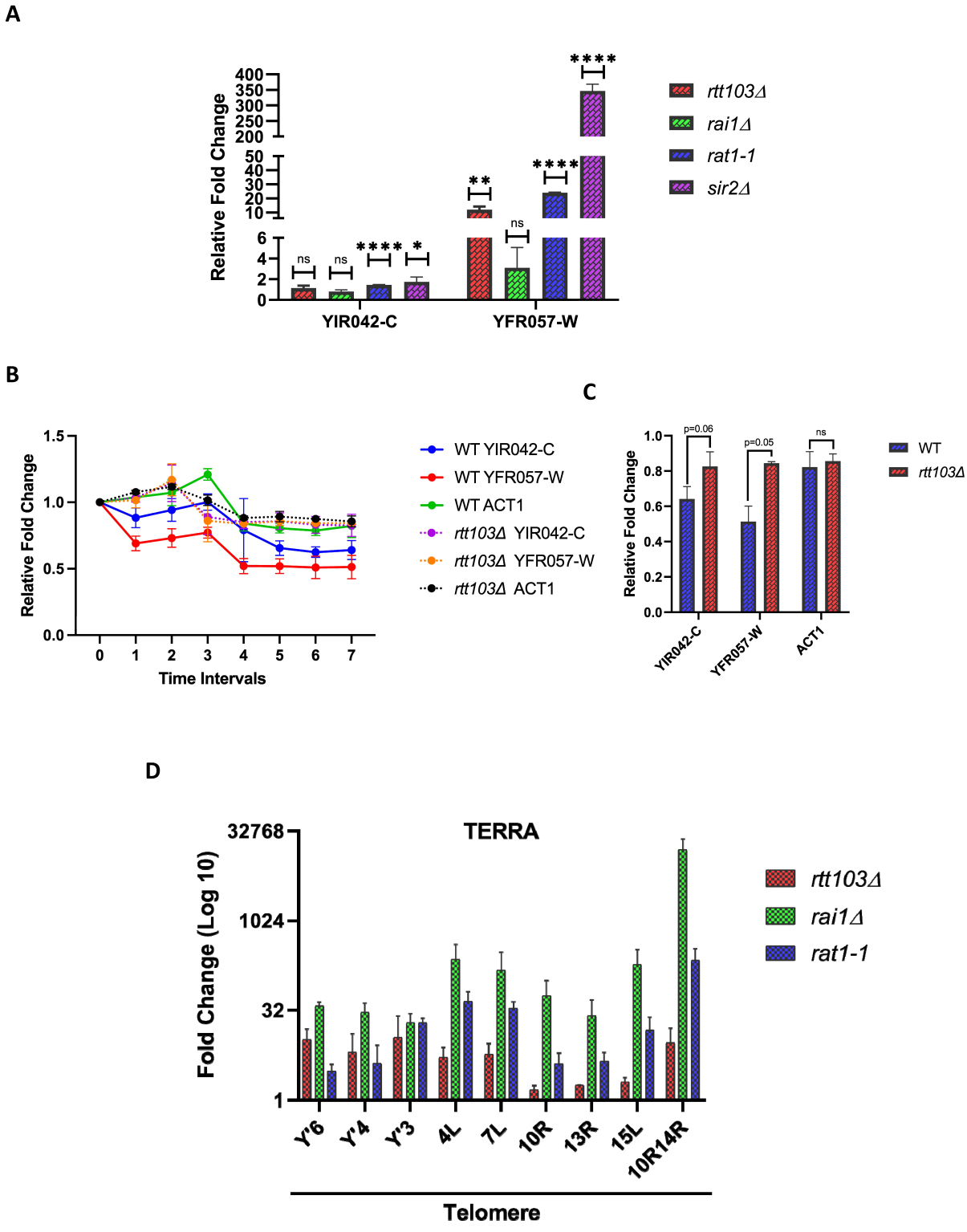

Figure 2. *Rtt103Δ, rai1Δ*, and *rat1–1* are defective in sub-telomeric silencing.

Note: A. Expression of YIR042-C and YFR057-W was assessed by qRT-PCR in the indicated strains. Fold change with respect to wild type is shown. All data are depicted as mean + SEM, n = 3. P values were obtained from student's t-tests.

B. *rtt103Δ* alters the stability of sub-telomeric transcripts. qRT-PCR analysis of the indicated sub-telomeric transcripts in *rpb1-1* and *rtt103Δ rpb1-1* after shifting to non-permissive temperature. RNA at the time 0-time point was arbitrarily set to 1. ACT1 was used as a control. The data from two independent experiments were plotted, and the error bars represent the standard deviation at each time point.

C. Relative abundance of YIR042-C, YFR057-W and ACT1 transcripts for the last time point was assessed in the indicated strains. All data are depicted as mean. P values were obtained from Student t-test (un-Paired).

D. TERRA accumulates in *rtt103Δ, rai1Δ and rat1-1*. RNA was isolated from the indicated strains and qRT-PCR with TERRA specific primers was performed to assess the levels in Y' and X-only containing telomeres. Three independent experiments were carried out, and an average of the three with standard error is plotted above. The primers Y'6/Y'4/Y'3/10R14R detect TERRA transcripts from more than one chromosomal end.

regulation at the telomeric regions [58–61]. In the RNA-Seq analysis of a *rtt103Δ* strain (Kathirvel and Mishra unpublished), we found several of the putative Y' helicases encoded within Y'-element of the sub-telomere region were up-regulated by more than twofold. In *S. cerevisiae* there are 11 Y′-Long elements and eight Y′-Short elements [11]. Each encodes more than one open reading frame (ORF) which potentially confers helicase activity [11,62]. In telomerase negative cells, these transcripts act as a template to alternatively lengthen the chromosome ends (ALT-Type I survivors) [10,11]. We first tested if the Y'helicases were indeed upregulated in the *rtt103Δ* strains via RT-PCR using single primer set to measure highly conserved Y' helicases from seven distinct loci. We find that Y' helicases are upregulated in *rtt103Δ* and *rai1Δ* (Fig S1B).

In addition to ORFs for putative helicases, the sub-telomeric region also contains promoter like elements for TERRA [28,63]. The TERRA sequence overlaps with the Y' helicases and ranges from ~100–1200 bases in size. TERRA is a major class of non-coding RNA that is produced from the sub-telomeric region and is conserved from yeast to humans [64]. In yeast, TERRA is an RNA Pol II product and has a 3' poly-A tail [27,30]. Its expression is tightly regulated via a Rap1p and Rat1p dependent mechanism and RNA surveillance pathways [12,27,30]. TERRA mostly remains associated at the telomeres suggesting a potential regulatory role in telomere replication and architecture [64,65] although precise functions of the TERRA transcripts still remains elusive. Therefore, we assessed the levels of TERRA from multiple chromosomes in an *rtt103Δ, rai1Δ* and *rat1–1* as described by Iglesias et al., 2011 via quantitative real-time PCR protocol [36] (Figure 2D, Fig S1C). We found that TERRA levels were increased in all the three mutants and *rai1Δ* had a larger level of accumulation compared to both *rtt103Δ* and *rat1-1*. While it is known that Rat1 plays a key role in keeping TERRA levels low, we find that both its partners, Rtt103 and Rai1, also contribute to regulating TERRA levels. These results suggest that, as proposed for transcription termination, the Rat1-Rai1 -Rtt103 could work together in the regulation of TERRA.

Rtt103p recruits Rat1p to telomeres in a transcription dependent manner

It has been reported that Rat1p associates with the telomeres during late S-phase when the telomere gets replicated and this association is dependent on the continued presence of Rif1 and Rif2 [12]. The association is abolished upon telomere shortening [12]. Moreover, recent work to identify telomere-associated proteins in *S. cerevisiae* via telomere-mimetic sequence as bait revealed both Rat1 and Rai1 are physically associated at telomeres [66]. In transcription termination, Rtt103 is proposed to be a recruiter for Rai1 and Rat1 by virtue of its interaction with RNA Pol II [43,67]. We, therefore, tested whether Rtt103 acts as a recruiter of Rat1 to the telomeres as well. We generated *rtt103Δ* in strains encoding TAP-tagged Rat1. TAP-ChIP was performed in Rtt103-TAP, Rat1-TAP and *rtt103Δ* Rat1-TAP strains and association with a few specific Y elements was measured. First, we found that Rtt103 is enriched at the telomeres to similar extents as that of Rat1. Second, in the absence of Rtt103, much lower levels of Rat1 could be detected (Figure 3A). As the Rat1 protein levels remain unaltered in an *rtt103Δ* (Figure 3B), it is the recruitment of Rat1 to the telomeres that is impaired significantly.

To further delineate whether this recruitment is transcription-dependent, we performed ChIP for Rat1 in the presence of thiolutin – a well-known inhibitor of yeast RNA polymerases [68]. Upon inhibition of transcription, there was substantial reduction in the enrichment of Rat1p at the most Y' regions tested (Figure 3C). We also performed ChIP with an already known point mutant of *RTT103* (R108N) which has reduced interaction with RNA Pol II as determined by anisotropy and NMR measurements [69]. The enrichment of Rat1-TAP at the telomeres was reduced in the strains harbouring *RTT103* (R108N) in comparison with the strain with wild type *RTT103* (Figure 3D) without any reduction in the total amount of Rat1-TAP protein in both strains. (Figure 3E). This suggests that it is the recruitment of Rat1p to telomeres is affected and strengthens the idea that atleast some Rat1 is recruited to telomeres via Rtt103 in a transcription-dependent manner.

Discussion

In this work, we demonstrate that Rtt103, a transcription termination factor, is required for efficient silencing of telomeric and sub-telomeric transcripts. The telomere length of *rtt103Δ* remains unaltered (Figure S1D). Similar to their role in transcription termination, both Rtt103 partners, Rai1 and Rat1, are also involved in regulating the levels of these transcripts. Of note, we show that Rtt103 could recruit Rat1, the exonuclease that that has been implicated in the degradation of these transcripts. We show that association of Rtt103 with the RNA polymerase II is required for regulating the levels of transcripts and that this association is likely mediated via interaction of the C terminal domain of RNA polymerase with Rtt103.

Several recent high-throughput transcriptional analyses have now established that transcription of telomeres and sub-telomeres is a conserved phenomenon among different phyla [27,29,32,63,70,71]. In humans, the TERRA length varies from few hundred to around 9Kb in size and is transcribed in a centromere to telomere orientation and most of the population is 7-methylguanosine (m7G) capped at the 5' ends while only 7% is polyadenylated [29]. In yeast, TERRA is transcribed from both Y' and X-only containing telomeres [36]. Their average size ranges from ~100–1200 bases and all are polyadenylated, while 5' m7G cap has not been demonstrated directly [72]. The TERRA transcripts mostly originate in the sub-telomeres suggesting a defined transcription start site. In humans, CpG islands on a sub-set of telomeres appear to be promoters and cytosine methylation at these sites negatively regulates transcription [28,63]. Since there appears to be defined initiation sites, the heterogeneity of TERRA size might be due to differential termination or processing of 3' ends. TERRA molecules lack the conserved poly-adenylation and cleavage signal 5′-AAUAAA-3′; and the mechanism of transcription termination remains elusive. In general, protein coding genes that contain the polyadenylation signal are terminated by the Rtt103-Rai1-Rat1 complex. However, for

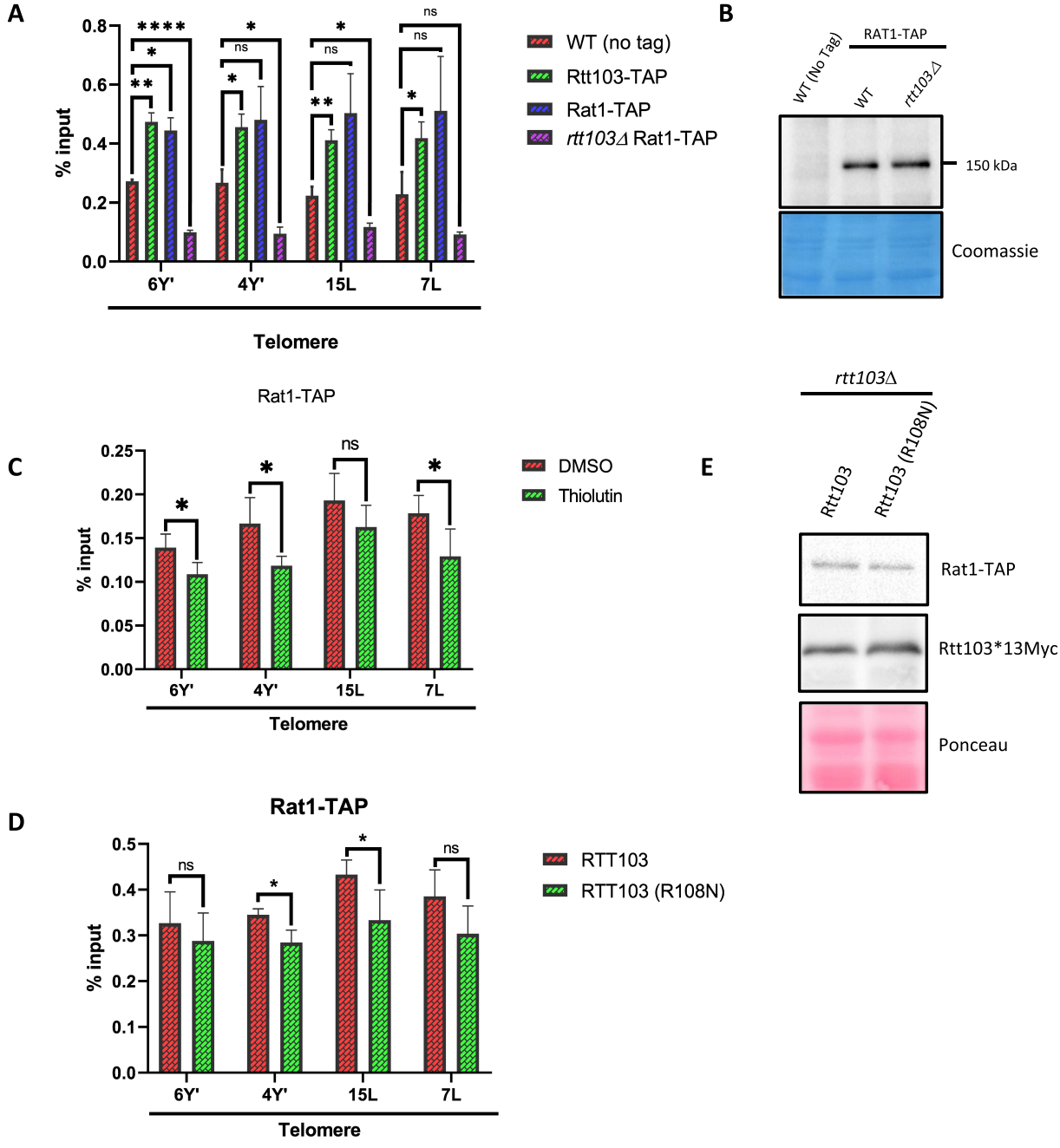

Figure 3. Rtt103p is physically associated with the telomeres.

Note: A. ChIP using IgG sepharose was performed with the indicated strains encoding TAP tags. Enrichment of Rtt103 and Rat1 at the indicated telomeres was analyzed. The WT without tag was used as a background control. The experiments were carried out three times, and the error bar represents the standard deviation. Statistical significance was calculated using two-way ANOVA (*$p < 0.05$).

B. Western blot analysis of expression of Rat1-TAP in WT and *rtt103Δ*. Rat1 protein levels remain comparable to WT in *rtt103Δ*. Blot was stained with Coomassie after developing as a loading control.

C. ChIP was performed for Rat1-TAP in the presence and absence of thiolutin. Enrichment at the TERRA/Y' loci was measured via qPCR and mean values were compared to their respective input. The experiment was done in triplicates and the mean was plotted. The error bars represent the standard deviation of mean and statistical significance was obtained using two tailed Student t-tests (*$p < 0.05$).

D. ChIP was performed for Rat1-TAP in an *rtt103Δ* harbouring ectopically expressed WT *RTT103* or the point mutant *RTT103 (R108N)*. Enrichment was measured via qPCR and mean values were compared to their respective input. All data are depicted as mean + SEM, n = 3. P values were obtained from two-tailed Student t-test (*$p < 0.05$).

E. Western blot analysis to assess the expression of Rat1-TAP in *rtt103Δ* harbouring Rtt103 (R108N). Ponceau stained blot serves as a loading control.

TERRA, earlier studies and our work suggest that the Rtt103-Rai1-Rat1 complex regulates stability and we speculate that termination mechanism may also be similar to that of protein coding genes.

In an *rtt103Δ*, silencing at the sub-telomeres is compromised at both modified and natural telomeres (Figure 1A–). All the three transcription termination mutants revealed varying degrees of silencing defects for the sub-telomeric genes, Y'

helicases and TERRA (Figure 2). Increasing evidence implying that Y' helicases and TERRA transcripts are being used as templates in telomerase negative cells makes us speculate this termination complex may have an important role to play in regulating telomere length in the absence of telomerase [11,12]. Although they work together in maintaining the levels of sub-telomeric transcripts, the variation in silencing might be due to impaired recruitment of Rat1 to the telomeres in case of *rtt103Δ* (Figure 3a), whereas in an *rai1Δ* mutant, it might be the compromised 5'-3' exonuclease activity exhibited by Rat1, as it has been reported that Rai1 enhances Rat1 activity in vitro [44]. In addition, Rai1 possesses a decapping, pyrophosphorylase and exonuclease activity as well and recognizes unmethylated Gppp caps [73–76]. We speculate that some of the transcripts may be degraded by the activity of Rai1 in a co-transcriptional manner before the RNA is fully capped and protected. This could also be why *rai1Δ* have increased TERRA compared to both *rtt103Δ and rat1–1*. Also decapping by Rai1 would expose a 5'phosphate that would make TERRA a substrate for Rat1.

In an *rtt103Δ*, the stability of the sub-telomeric transcripts is specifically altered, implying that these transcripts might be co-transcriptionally regulated in a Rat1-dependent manner (Figure 2b,c). Here we report that the enrichment of Rat1 to the telomeres is via Rtt103 in a transcription-dependent manner (Figure 3c). Previous studies have reported that when a telomere gets shortened, the association of Rat1 to the telomere is abolished and the continued presence of Rif1 and Rif2 is required for the association [12]. The absence of Rat1 at the telomeres in *rif1* and *rif2* could be due to the increased TPE and hence inaccessibility to the transcription machinery [77,78]. Alternately, Rat1 could be recruited independently by both Rap1/Rif1/Rif2 and Rtt103 to telomeres. It is known that TERRA exists in three different fractions, namely, chromatin associated, nucleoplasmic and cytoplasmic fraction [79]. It has been suggested that the non-chromatin associated fraction is regulated via degradation by Rat1 [12]. Here in this study, we report the regulation of TERRA levels by the Rtt103-Rai1-Rat1 termination complex and propose a mechanism by which Rat1 may be targeted to the TERRA RNA.

How might Rtt103 regulate sub-telomeric transcript levels? We envision multiple possibilities (Figure 4a and b). At the X elements where Sir-dependent silencing is robust, we propose that it is the escape transcription that is regulated by this complex as proposed earlier [80]. Normally, Sirp-dependent epigenetic silencing silences much of the transcription; any transcription that is initiated is prematurely terminated and if transcription is completed then it is subjected to exosomal degradation. We suggest the improper termination of transcription at the inserted *URA3* locus leads to production of transcripts with longer 3' ends that might be poor substrates for exosomes, leading to export of this transcript and translation. At the non-coding TERRA site, one possibility is the co-transcriptional recruitment of Rat1 (via Rtt103) to the sub-telomeric transcripts leads to degradation. As Rat1 can only

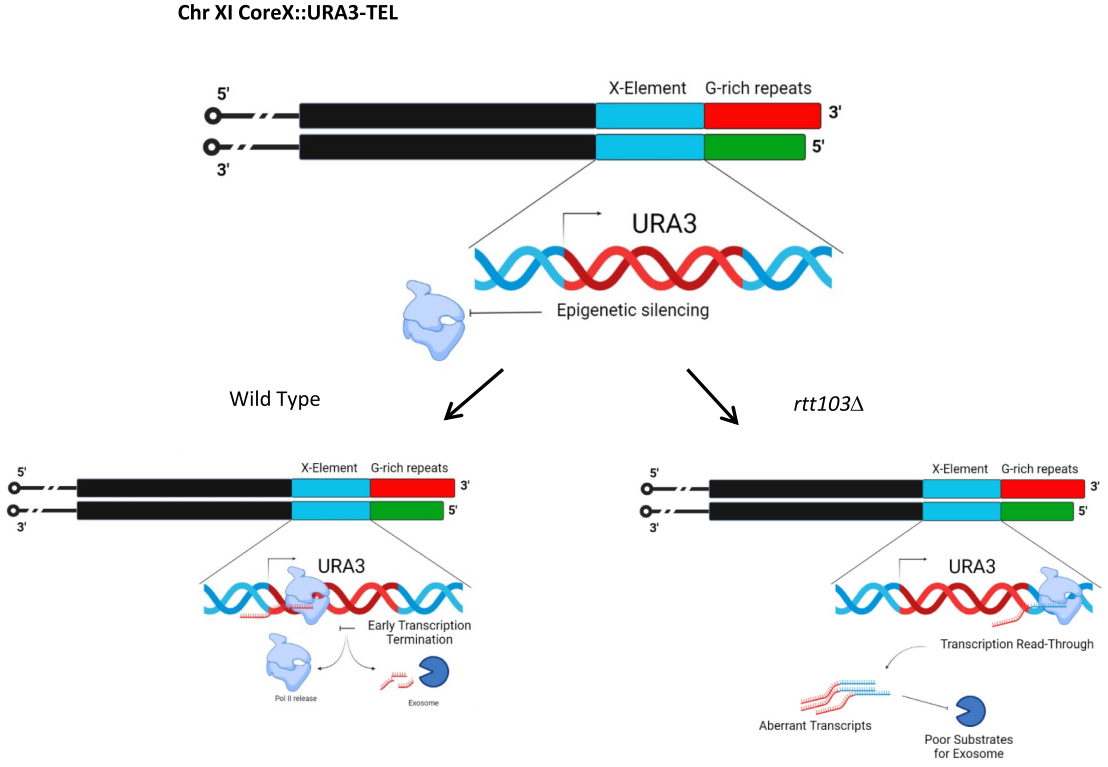

Figure 4a. Silencing at X-element heterochromatic loci: In a wild type heterochromatic loci are kept repressed via three independent mechanisms. 1. Epigenetic silencing – Heterochromatin formation and repression of RNA Pol II via SIR complex (major pathway). 2. Premature termination of transcription 3. Any leaky mature transcripts will be targeted for exosome mediated degradation. In an *rtt103Δ* we speculate that there could be transcription read-through due to impaired recruitment of Rai1 and Rat1 for proper termination. As such aberrant transcripts are poor substrates for exosome mediated degradation they exhibit increased stability in *rtt103Δ*.

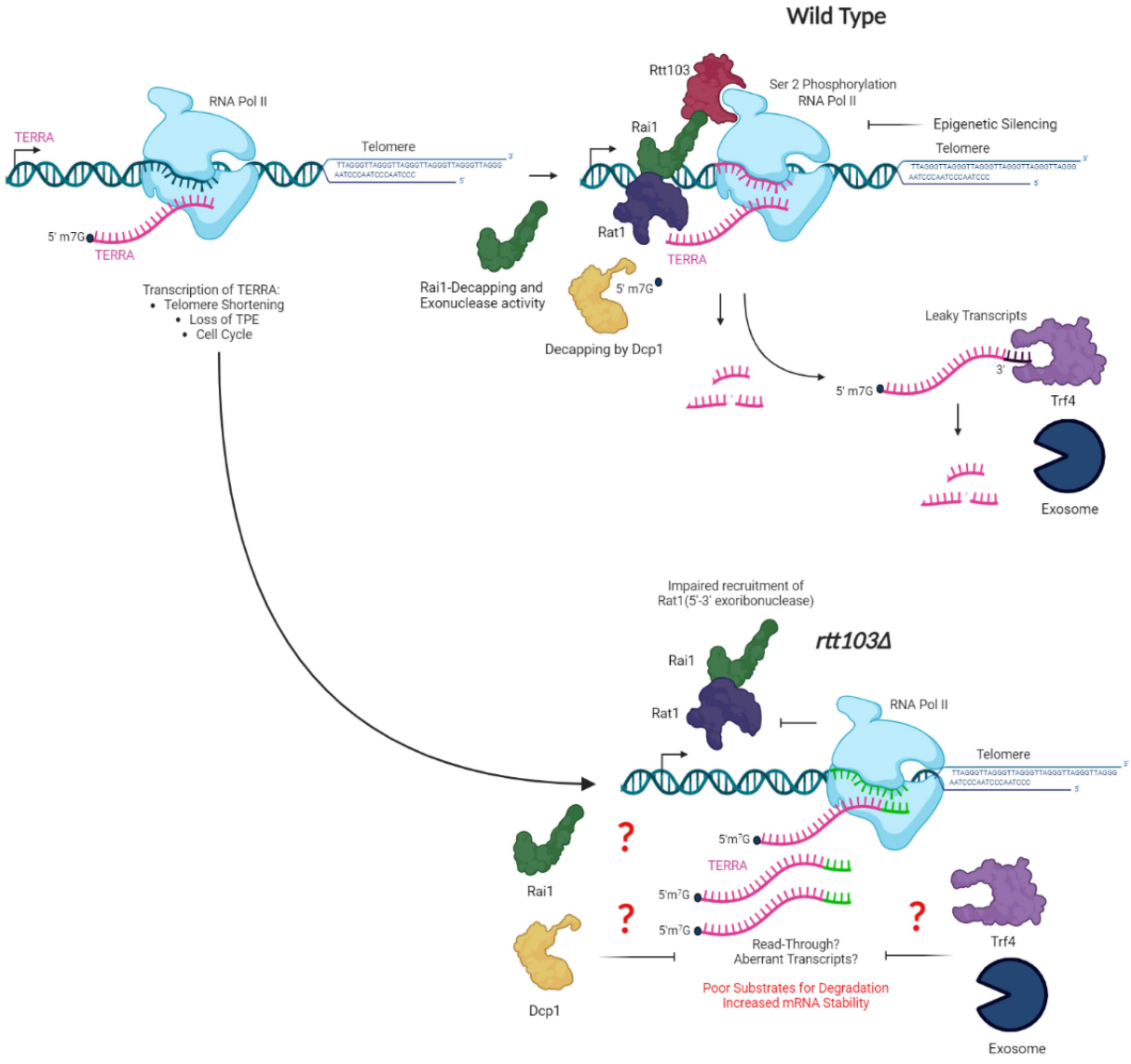

Figure 4 b. Co-Transcriptional regulation of TERRA : In a wild type, the TERRA levels are kept repressed via Rap1 through Rif1/2 and Sir2/3/4 complex and by Rat1 exonuclease activity. A minor pathway that regulates TERRA is the post-transcriptional degradation via TRAMP-mediated exosome targeting. In our study, we speculate there may be co-transcriptional regulation of TERRA by the Rtt103-Rai1-Rat1 complex. Rtt103 recruits Rai1 and Rat1 in a RNA polymerase-dependent manner to the TERRA locus a) where Rai1 exhibits its pyrophosphohydrolyase activity towards mRNA lacking 5′-end cap and perhaps prepares the substrate for Rat1 mediated exonuclease activity. Absence of Rtt103 leads to impaired recruitment of Rai1 and Rat1 resulting in reduced Rat1 mediated degradation of TERRA. It also potentially leads to read-through transcripts which are poor substrates for exosome-mediated degradation.

act on uncapped 5' ends, we think that once the RNA is capped, it has to be decapped and then Rat1 can degrade. This is possibly post-transcriptional. Alternately or additionally, Rai1 could also independently exhibit this activity on nascent transcripts co-transcriptionally once recruited to the transcribing polymerase as it possesses a decapping and exonuclease activity on unmethylated 5' caps [73–76]. As loss of Trf4, which is involved in targeting RNA to nuclear exosome, also increases TERRA abundance (albeit a minor one), it is possible atleast some of the TERRA is targeted to the nuclear exosome. We suggest that the TERRA transcripts produced in the absence of Rtt103-Rai1-Rat1 may have abnormal 3' ends and may not be degraded by the exosome machinery efficiently leading to increased accumulation of these transcripts [81]. In sum, we propose that free TERRA is kept at very low levels in wild type by the combined action of Rtt103, Rai1 and Rat1 by targeting it in a co-transcriptional manner.

Interestingly, Rtt103 was initially isolated in a screen for mutants that elevated Ty1 transcription and it was demonstrated that there was a moderate increase in Ty1 transcripts in *rtt103Δ* [82]. This raises the intriguing possibility that Rtt103 could be involved in negatively regulating Ty1 transcripts and in its absence, Ty1 transcripts are stabilized leading to increased cDNA and increased transposition. In another possible link, Y' helicase transcripts are also higher in *rtt103Δ* and it is known that Y' helicase is incorporated into the viral like particles produced in the Ty1 transposition cycle [10]. Together these

observations suggest a potential role for RTT103-mediated RNA stability in processes affecting transposition and hence genome stability.

There are a number of key questions that remain to be addressed. Induction or overexpression of TERRA from a single telomere induces early-onset senescence [83]. Does stabilization of TERRA also have similar effects? Is transcription of TERRA a coordinated event from all the telomeres? In the case of sub-TERRA and ARIA, it is transcribed from telomere end towards the centromere. Do the telomere ends also possess promoter like elements? In *S. cerevisiae,* sub-TERRA XUT and sub-TERRA CUT are complementary to each other and can form dsRNA. As RNAi does not exist in *S. cerevisiae*, could RNA degradation pathways be more critical to regulate the levels of such non-coding telomere transcripts?

The sub-telomeric regions reveal higher levels of recombination leading to faster evolution of gene families residing in this locus [84–86]. This allows faster and better adaptive responses to the changing environment. Additionally, it contributes to antigenic variation and virulence in some pathogenic yeasts and parasites [28,87–89]. Therefore, understanding regulation of telomeric and sub-telomeric transcription has implications beyond yeast. While this study shows which transcription termination complex involved in such regulation, a study of biological cues which decide between maturation or co-transcriptional degradation might provide effective ways to inhibit transcription of genes which are sub-telomeric in origin. The sub-telomeric region also harbours various other classes of genes like *tlh* which is regulated under nitrogen starvation, cri-TER a temperature-dependent non-coding RNA, genes responsible for biofilm formation [90–92]. It remains to be investigated if expression of these genes are also regulated post-transcriptionally.

List of strains and plasmids used in this study

The *Saccharomyces cerevisiae* strains used in this study are derivatives of W303 or BY4741 and are listed in Table 1. Strains were grown under standard conditions in YPD (Yeast Peptone Dextrose) or synthetic complete (SC) medium at 28°C. For temperature-sensitive (*ts*) mutants, further specifications are mentioned in the methods section. Standard procedures were followed for yeast manipulations. Either micromanipulation or PCR-based homology-dependent transformation was employed to generate knock-outs [93].

Methods

Yeast spot growth assay

For spotting assays, yeast cells were grown overnight at 28°C in appropriate selection media. Cells were harvested at OD_{600} – 1 and ten-fold serial dilutions were made and 5 µl spotted onto appropriate agar plates. Plates were then incubated for 2–3 days at 28°C and photographed. The FOA concentration used is 1 mg/ml. For the *ADE2* colour-based silencing assay, cells were directly plated on YPD agar plates, incubated at 30°C for 2 to 3 days and photographed.

RNA preparation and c-DNA synthesis

Total RNA was isolated by extraction with hot acidic-phenol (pH-5) as described by Collart and Oliviero [94]. For RNA half-life experiments, cells were grown in SC medium- to mid-log phase at permissive temperature (25°C), centrifuged and shifted rapidly to non-permissive temperature (39°C) by adding equal amount of pre-warmed medium (50°C) and incubated at 39°C for the respective intervals. For *rat1–1 ts* mutant, the cells were sub-cultured and grown up to OD_{600} 0.5 ~ 0.8 (28°C) and shifted to non-permissive temperature (39°C) for 3 hours. For data normalization of *rat1–1*, similarly treated wild-type cells were used. For DNaseI digestion, 3 µg of RNA was subjected to digestion with 10 units of DNaseI (New England Biolabs) enzyme at 37°C for 3.5 hours to

Strain No.	Genotype	Source
KRY 105	W303 *adh4::ADE2* Tel VII L MAT-a	Lab collection [50]
KRY 193	W303 *adh4::URA3* Tel VII L MAT-a	Lab collection [50]
KRY171	yku70:KanMx adh4:Ade2 rad5+mat a	Lab collection [50]
KRY 230	KRY 105 except *rtt103Δ*::KanMx MAT-α	Lab collection [50]
KRY 285	KRY 193 except *rtt103Δ*::KanMx MAT-α	Lab collection [50]
KRY 172	KRY 193 except *yku70Δ*::KanMx MAT-α	Lab collection [50]
KRY 632	*rai1Δ*::KanMx *his3Δ1 leu2Δ0 ura3Δ0* MAT-α	Arlen Johnson
KRY 634	*rat1–1^{ts} ura3–52 leu2Δ1 his3Δ200 trp1Δ63* MAT-α	Arlen Johnson
KRY 2187	yRP693 *ura3–52 leu2 rpb1–1^{ts}*	Carolyn Decker
KRY 2188	KRY 2187 except *rtt103Δ*::KanMx	This Study
KRY 2189	BY4741 RTT103-TAP:HIS3Mx6	Horizon Discovery
KRY 2190	BY4741 RAT1-TAP:HIS3Mx6	Horizon Discovery
KRY 2191	KRY 2190 except *rtt103Δ*::KanMx MAT-α	This Study
KRY2234	KRY105 except *rtt103Δ*::KanMx *rai1Δ*::KanMx MAT-α	
KRY 931	YEF505 ura3, leu2, ade2Δ, telXI coreX:URA3, SIR3-GFP:TRP1 MATα	Emmanuelle Fabre [55]
KRY 2192	KRY931 except *rtt103Δ*::KanMx	This Study
CKM 261	Full length RTT103 in YCplac22	Lab collection [50]
CKM 285	Full length RTT103 in YEplac112	Lab collection [50]
CKM 287	RTT103*13xMyc in pBEVY-T	This study
CKM 767	RTT103 (R108N) *13Myc in pBEVY-T	This Study

achieve an RNA which is completely devoid of telomeric DNA. Before c-DNA synthesis, a normal PCR (40 Cycles) was performed with primers specific for telomere and *ACT1* to assess the DNA contamination. RNA alone was also employed as a template for qRT-PCR to confirm the purity of the samples. The reverse transcription was performed using Verso cDNA Synthesis Kit (Thermo Scientific) as per the manufacturer's protocol at 55°C for 60 min followed by inactivation at 95°C for 2 min.

TERRA level analysis by qRT-PCR

TERRA reverse transcription was done using 10 µM CA oligonucleotide and 2 µM *ACT1* oligonucleotide in a final volume of 20 µl. For qPCR, the cDNA was diluted with equal volume of nuclease-free H_2O. A volume of 1 µl cDNA was quantified in a final volume of 10 µl reaction by real-time PCR with the Power SYBR Green PCR Master mix (Applied Biosystems) using an Quanstudio3 Real-Time PCR System. The final concentration for each primer set differs from 0.2 to 0.6 µM as described by Iglesias *et al.*, 2011 [36]. The reactions were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 59°C. TERRA levels were normalized to respective actin values and compared to the isogenic wild type. The primers Y'6/Y'4/Y'3/10R14R detects TERRA stemming from more than one chromosomal end (6Y'- 8 L/8 R/12 L-YP1[a] /12 R-YP2[a] /13 L/15 R) (4Y'-9 L/10 R/12 R-YP2[a] /15 R) (Y'3-12 L-YP1[a] /12 R-YP2 [a] /15 R). Whereas for X-only containing telomeres, individual primers have been designed – 4 L, 7 L, 10 R, 13 R, 15 L, 10R14R.

Chromatin immunoprecipitation

ChIP was performed as described [95]. Briefly, yeast cells were grown to OD_{600} 0.8–1 and crosslinked for 10 mins with formaldehyde (final conc. 1.2%) and quenched with glycine (360 mM) for 15 mins. Cells were pelleted and washed twice with 1× TBS, resuspended in lysis buffer (0.1% deoxycholic acid, 1 mM EDTA, 50 mM HEPES/KOH pH-7.5, 140 mM NaCl, 1% Triton X-100, protease inhibitor cocktail) and lysed using 0.5-mm glass beads in a vortex mixer for 20 min at 4°C. The chromatin lysate was recovered and sheared 10 sec on/off (Henderson Biomedical MSE/Amplitude 10) for 5 cycles. An input sample representing 10% of the ChIP extract was employed as input for normalizing the qPCR. 80 µl bed volume of IgG Sepharose beads were washed with lysis buffer and IP was performed overnight at 4°C. Beads were washed with lysis buffer P500 (0.1% Deoxycholic acid, 1 mM EDTA, 50 mM HEPES/KOH pH7.5, 500 mM NaCl, 1% Triton X-100), + LiCl detergent buffer (0.5% deoxycholic acid, 1 mM EDTA, 250 mM LiCl, 0.5% NP-50, 10 mM Tris-Cl pH8) + 2× TBS. The bead-bound DNA was eluted in 100 µl (1% SDS/1× TBS) + 150 µl (1% SDS/1× TBS) for 10 min at 65°C. For reversing the crosslink, input and IPs were treated overnight at 65°C with proteinase K and RNase A. DNA from bound (IP) and unbound fraction (input) was purified by Phenol-Chloroform-Isoamyl alcohol (PCI- 25:24:1) extraction followed by ethanol precipitation. qPCR was performed as mentioned above and % input calculation was employed to assess the relative enrichment.

SDS-PAGE and western blot

Cells corresponding to 2 units of OD_{600} were subject to protein extraction by TCA method. To the cell pellet 200 µl of 20% TCA was added and lysed by vortex at high speed for 5 min. The lysate was then collected onto a fresh tube and the glass beads were washed twice with 150 µl of 5% TCA. The washed elutes were added to the previous lysate and spun at 13000 rpm for 10 min. To the pellet 200 µl of Laemmli buffer was added and pH was adjusted using 2 M Tris.

The dissolved protein pellet was then denatured by boiling at 95°C for 5 min. SDS-PAGE was performed followed by a semi-dry method of transfer (Power Blotter system/Invitrogen) to PVDF membrane for western blotting. The blots were then probed with primary TAP-tag antibody (Genescript A01435, 1:5000) and HRP conjugated secondary anti-rabbit (Abcam ab97051, 1:10,000). For probing Rtt103 × 13myc, rabbit Myc (Abcam Ab9106 1:1000) primary was used. The signal was detected by ECL reagent (BioRad) and imaged in ChemiDoc Imaging system by BioRad.

Northern hybridization by slot blotting and southern

For RNA slot blots, 10 µg of denatured RNA was loaded onto Hybond N+ membranes (Amersham) using slot blot manifold Hoefer PR648 (Amersham). For southern blot, 3 µg of overnight digested XhoI genomic DNA was loaded. After transfer, blots were auto cross-linked via Stratagene UV Stratalinker 1800. The probes and whole hybridization procedure were carried out according to the manufacturers instruction for DIG-High prime DNA labelling and detection starter kit I (Roche 11,745,832,910). For Tel probe, terminally digoxigenin labelled $d(GT)_{30}$ was used.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Author contributions

KR contributed to the acquisition of data, analysis, and interpretation of data. KM contributed to the conception and design, analysis and

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Declaration

The authors declare that they have no competing interests.

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